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May 2010

Respiratory Infections

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On the Cover

Judith Leyster (1609–1660)
Boy Playing The Flute (1630–1635)
Oil on canvas (73 cm × 62 cm)
The National Museum of Fine Arts, Stockholm, Sweden

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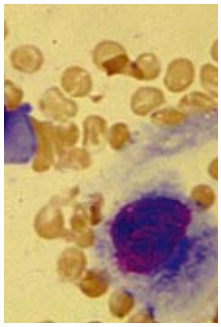

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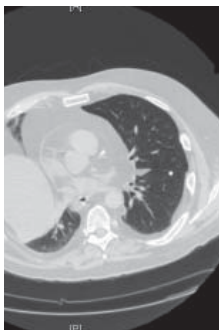

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Conference Summary

Latent Tuberculosis among Persons at Risk for Infection with HIV, Tijuana, Mexico

Richard S. Garfein, Rafael Laniado-Laborin, Timothy C. Rodwell, Remedios Lozada, Robert Deiss, Jose Luis Burgos, Jazmine Cuevas-Mota, Paris Cerecer, Kathleen Moser, Maria Luisa Volker, and Steffanie A. Strathdee

Because there is little routine tuberculosis (TB) screening in Mexico, the prevalence of latent TB infection (LTBI) is unknown. In the context of an increasing HIV epidemic in Tijuana, Mexico, understanding prevalence of LTBI to anticipate emergence of increased LTBI reactivation is critical. Therefore, we recruited injection drug users, noninjection drug users, female sex workers, and homeless persons for a study involving risk assessment, rapid HIV testing, and TB screening. Of 503 participants, the overall prevalences of TB infection, HIV infection, and TB/HIV co-infection were 57%, 4.2%, and 2.2%, respectively; no significant differences by risk group ($p>0.05$) were observed. Two participants had TB (prevalence 398/100,000). Incarceration in Mexico (odds ratio [OR] 2.28), age (OR 1.03 per year), and years lived in Tijuana (OR 1.02 per year) were independently associated with TB infection ($p<0.05$). Frequent LTBI in marginalized persons may lead to increases in TB as HIV spreads.

Tuberculosis (TB) is endemic to Mexico. The national TB incidence is estimated to be 16.2 cases/100,000, but regional rates, particularly along the United States–Mexico border are much higher (*1*). Baja California, which shares a border with California, has the highest incidence of pulmonary TB in Mexico (57 cases/100,000), which is >3× the national average (*2*). Furthermore, California and Baja California have the highest incidence rates of all the border states in their respective countries (*1*). Transmission of TB from high-prevalence countries to low-prevalence countries, such as the United States, poses a major public health concern. Tijuana, the largest city in Baja California, Mexico, lies ≈20 miles south of downtown San Diego, California, and these 2 cities are loosely separated by the busiest land border crossing in the world, with >90,000 passenger vehicles crossing in both directions daily (*3*). As a possible consequence of this close binational association, San Diego has a slightly higher incidence of TB than California (8.4 per 100,000 and 7.0 per 100,000, respectively) (*4*,*5*).

Otherwise healthy persons with dormant or latent tuberculosis infection (LTBI) have a 10% lifetime risk that dormant mycobacteria will become active and cause TB. Persons co-infected with HIV and LTBI have a risk for TB reactivation of 10% per year (*6*). Consequently, TB is a leading cause of death worldwide among persons co-infected with TB and HIV (*7*). Although HIV prevalence in Mexico is lower than that in the United States, Tijuana is currently experiencing an emerging HIV epidemic (*8*), with increasing prevalence observed among high-risk groups such as injection drug users (IDUs) and female sex workers (FSWs) (*9*–*11*). The potential for rapid HIV transmission among IDUs in Mexico is highlighted by findings from Tijuana and Ciudad Juarez (a border city south of El Paso, Texas), which showed that 95% of IDUs in these cities had antibodies against hepatitis C virus (a marker of unsafe injection practices), and most IDUs had self-reported risk factors for sexual or parenteral exposure to HIV (*12*).

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Thus, there is concern that increased HIV incidence would result in LTBI reactivation among IDUs who spread active TB to other populations, which would lead to a more generalized epidemic.

Homeless persons and noninjecting drug users (NIDUs) are also at increased risk for co-infection with TB/HIV. In low-prevalence countries, these risk groups have higher rates of LTBI than those in the general population (*13*–*15*). Although estimates of LTBI and their correlates among FSWs are not well documented, an increasing percentage of FSWs in Tijuana are infected with HIV and report multiple potential risk factors for TB, including injection drug use and sexual contact with IDUs (*10*). Thus, the need for accurate estimates of LTBI prevalence in high-risk marginalized populations is clear.

In the United States, LTBI screening and prophylactic treatment have played a major role in reducing co-infection with TB/HIV in most regions and communities. However, LTBI screening, which is usually conducted by using the tuberculin skin test (TST), is uncommon in countries such as Mexico, which still uses *Mycobacterium bovis* BCG vaccination universally, because the TST has reduced specificity in vaccinated persons (*16*). Whole-blood interferon-γ release assays (IGRAs), which measure cellular immune response to purified proteins found in *M. tuberculosis,* but not in BCG vaccine strains, provide a means for more accurately estimating TB infection prevalence in countries such as Mexico (*17*). A study that measured TB infection prevalence in Mexico by using an IGRA found that 67% of IDUs in Tijuana were positive (*18*). However, that study did not differentiate between LTBI and active TB and included only IDUs. The purposes of the current study, known as PreveTB, were to measure the prevalence of TB and HIV among marginalized populations of Tijuana who are at a high risk for becoming co-infected with HIV and TB, estimate the prevalence of active TB in this group, and identify correlates of LTBI.

Methods

Study Population

Participants were recruited in Tijuana, Mexico, during April 2007–July 2007, by street outreach, targeted advertising, and word-of-mouth. Persons were eligible to participate if they were ≥18 years of age, provided informed consent, planned on staying in Tijuana for the next 30 days, and reported at least 1 of the following characteristics in the 6 months before enrollment: use of noninjected illicit drugs other than marijuana, injection drug use, receipt of money/goods in exchange for sex, and homelessness or unstable housing. Unstable housing was defined as living primarily in a rented hotel room, migrant work camp, or medical/drug treatment facility.

Data Collection

Participant interviews were conducted and biological samples were obtained at PrevenCasa, a community-based harm-reduction and research facility located in the Zona Norte neighborhood of Tijuana, which abuts the commercial sex trade district. Computer-assisted personal interviewing technology (QDS; Nova Research Company, Bethesda, MD, USA) was used to facilitate participant interviews. Interviews were conducted in Spanish by trained Mexican interviewers experienced with the specific populations. Questions included sociodemographic characteristics, putative risk factors for TB and HIV infection, TB knowledge and exposure history, and presence of TB-related symptoms. The survey instrument was developed in English, translated into Spanish, and then back-translated into English to verify accuracy and meaning. Monetary reimbursement of US $20 was offered to participants to compensate them for time and transportation.

During the computer-assisted personal interview, persons who reported a persistent cough for ≥3 weeks and an additional symptom indicative of TB (fever or chills, night sweats, swollen lymph nodes, hoarseness, shortness of breath, joint pain, fatigue, and unexplained weight loss) or hemoptysis with or without other symptoms, were considered suspected active TB case-patients. After the interview, these persons were asked to provide 3 sputum samples for acid-fast bacilli (AFB) smears; the first of these samples was collected immediately. The remaining 2 samples were collected on subsequent days. These participants were also transported to a nearby radiologic center for chest radiography to identify evidence of pathologic changes consistent with TB. Incentives of US $5 were given for each additional sputum collection visit and upon completion of a chest radiograph, as recommended by FitzGerald et al. (*19*). Participants with ≥1 AFB-positive sputum smear or chest radiography findings consistent with TB were determined likely to have active TB and were referred to the central public health clinic (Instituto de Servicios de Salud Pública del Estado de Baja California [ISESALUD]) for clinical confirmation and treatment through the national TB program. The study protocol was reviewed and approved by ethics committees at the University of California, San Diego, and the Tijuana General Hospital.

Laboratory Testing

M. tuberculosis infection was detected by using an IGRA (QuantiFERON TB Gold In-Tube [QFT] assay; Cellestis Ltd., Carnegie, Victoria, Australia), an in vitro assay that uses an ELISA to detect interferon-γ released by whole blood samples after introduction of 6-kDa early secretory antigenic target protein, culture filtrate protein 10, and TB7.7 protein, which mimic antigens specific to the *M. tuberculosis* complex present in patients with TB and

LTBI (*20*). In 2005, the US Food and Drug Administration (Silver Spring, MD, USA) approved the QFT assay for detection of *M. tuberculosis* infection, and the US Centers for Disease Control and Prevention (Atlanta, GA, USA) determined that this assay may be used to detect *M. tuberculosis* in all situations in which the TST is used (*17*). Antibodies against HIV were detected by using the Determine Rapid HIV Antibody Test (Abbott Laboratories, Boston, MA, USA) on site. All positive test results were confirmed at the San Diego County Public Health Laboratory by using an ELISA and an immunofluorescent antibody assay.

After data collection, all persons who did not report symptoms consistent with active TB were given an appointment 4 weeks later to receive the results of the HIV test and QFT assay. At this visit, those who were HIV positive were referred to ISESALUD for medical evaluation, and those who were IGRA positive were offered chest radiography when they returned to obtain results that ruled out active TB. Persons who had active TB by AFB smear or radiography were referred to ISESALUD. Persons who were IGRA positive, but had an unremarkable chest radiograph, were determined to have LTBI.

Statistical Analysis

Descriptive statistics were used to characterize participants by IGRA status. Associations between participant characteristics and TB infection were evaluated by using the Wilcoxon rank-sum test or Fisher exact test, followed by univariate and multivariate logistic regressions. Variables with p values <0.10 in univariate models were considered for inclusion in multivariate analysis. A backward stepwise model that selected for main effects in the final model was used. At each step, likelihood ratio testing was used to compare nested models until only variables with p values <0.05 remained in the final model.

Results

Prevalence of HIV and TB Infection

A total of 527 persons were recruited, of whom 503 met eligibility criteria and had complete HIV and IGRA results. Overall, 57% were positive for TB infection by QFT assay (IGRA+), 4.2% were HIV+ by HIV test, and 2.2% were positive by both tests. Fifty-nine percent of participants were men, median age was 36 years (interquartile range 29–42 years), and median length of time participants had lived in Tijuana was 6 years (interquartile range 1.0–13.3 years). On the basis of nonmutually exclusive groupings, there were 232 (46%) IDUs, 311 (62%) NIDUs, 115 (23%) FSWs, and 280 (56%) homeless persons. The prevalence of IGRA+ results was 63% among IDUs, 58% among NIDUs, 49% among FSWs, and 52% among homeless participants. A total of 14 (2.8%) participants reported symptoms suggestive of active TB, of which 2 persons were diagnosed with active TB by AFB+ smears (prevalence 398/100,000). Because chest radiography was used after recruitment began and was offered at the results visit, only 79 of the 286 IGRA+ participants had chest radiography, of which 8 (10%) had signs of current or past pulmonary TB.

Correlates of TB Infection

Univariate analysis identified several factors associated with TB infection (Table 1). When compared with IGRA– participants, IGRA+ participants were significantly older (median age 38 vs. 34 years; p<0.01), had lived in Tijuana longer (median 9.0 vs. 3.5 years; p<0.01), were more likely to be men (64.7% vs. 51.6%; p<0.05), had stable sleeping arrangements in the 6 months before data collection (49.3% vs. 37.8%; p<0.05), and had known someone with TB (46.2% vs. 35.9%; p<0.05). When compared with those not in each risk group, IDUs were more likely to be IGRA+, and FSWs and homeless participants were less likely to be IGRA+; no association was found for NIDUs. History of incarceration was associated with TB infection. Persons who had been incarcerated in Mexico were more likely to be IGRA+ (68%) than those incarcerated in the United States (55%) or both countries (62%). To maximize statistical power, in subsequent analyses we combined incarceration in Mexico and the United States into 1 incarceration category. When compared with IGRA– participants, IGRA+ participants were no more likely to report sexually transmitted infections (2.5% vs. 2.3%; p = 0.91) or be HIV+ (3.8% vs. 4.6%; p = 0.67). TB infection was not associated with any other drug administration practice or sexual behavior examined in this study.

Multivariate logistic regression analysis (Table 2) showed that TB infection was independently associated with age, increasing years of Tijuana residence, and a history of being incarcerated in Mexico or in Mexico and the United States. For each 1-year increase in age and time lived in Tijuana, participants were 1.03× (95% confidence interval [CI] 1.01×–1.05×) and 1.02× (95% CI 1.01×–1.04×) more likely to be IGRA+, respectively. Persons who had a history of being jailed in Mexico were 2.28× (95% CI 1.48×–3.51×) more likely to be IGRA+ than those who had never been incarcerated.

Discussion

We found a high prevalence of TB infection among marginalized populations at high risk for HIV infection in Tijuana. Although HIV prevalence in this study (4.2%) was lower than estimates reported among similar populations elsewhere (*21*,*22*), it was higher than that of the general population of Baja California, Mexico (0.8%–0.9%) (*9*,*23*). If HIV prevalence increases among groups who have high LTBI prevalence, reactivation and spread of TB will ham-

Table 1. Univariate analysis of factors associated with tuberculosis infection status among high-risk groups for HIV in Tijuana, Mexico, April–July 2007*

Characteristic	Total (n = 503)	IGRA– (n = 217)	IGRA+ (n = 286)	Odds ratio (95% CI)
Risk factor†‡				
Injected drugs	232 (46.1)	86 (39.6)	146 (51.1)	1.59 (1.11–2.27)
Used drugs but never injected	207 (41.2)	94 (43.3)	113 (39.5)	0.86 (0.60–1.22)
Sex work	115 (22.9)	59 (27.2)	56 (19.6)	0.65 (0.43-0.99)
Homeless/unstably housed	280 (55.7)	135 (62.2)	145 (50.7)	0.63 (0.44–0.90)
Median age, y (IQR)	36.0 (29–42)	34.0 (28–40)	38.0 (31–43)	1.04 (1.02–1.06)
Sex				
M	297 (59.0)	112 (51.6)	185 (64.7)	1.00
F	193 (38.4)	99 (45.6)	94 (32.9)	0.58 (0.40–0.83)
Transgender	13 (2.6)	6 (2.8)	7 (2.5)	0.71 (0.23–2.16)
Years lived in Tijuana, median (IQR)	6.04 (1–13.3)	3.51 (0.3–10.0)	9.0 (3.0–16.4)	1.03 (1.012–1.04)
Most frequent type of sleeping arrangement‡				
Stable	223 (44.3)	82 (37.8)	141 (49.3)	1.00
Unstable housing	209 (41.6)	105 (48.4)	104 (36.4)	0.58 (0.39–0.85)
Homeless	71 (14.1)	30 (13.8)	41 (14.3)	0.80 (0.46–1.37)
Ever incarcerated	338 (67.2)	128 (59.0)	210 (73.4)	1.92 (1.32–2.80)
Incarceration status				
Never jailed	165 (32.8)	89 (41.0)	76 (26.6)	1.00
Jailed in USA only	129 (25.6)	58 (26.7)	71(24.8)	1.43 (0.90–2.28)
Jailed in Mexico or Mexico and USA	209 (41.5)	70 (32.3)	139 (48.6)	2.33 (1.53–3.54)
Noninjection drug used most often‡				
None	157 (31.2)	67 (30.9)	90 (31.5)	1.00
Methamphetamines	218 (43.3)	101 (46.5)	117 (40.9)	0.86 (0.57–1.30)
Heroin	52 (10.3)	14 (6.5)	38 (13.3)	2.02 (1.01–4.03)
Any other drug	76 (15.1)	35 (16.1)	41 (14.3)	0.87 (0.50–1.51)
Ever injected illegal drugs	248 (49.3)	92 (42.4)	156 (54.6)	1.63 (1.14–2.33)
Mean (SD) years injected drugs among IDUs	13.5 (13.0)	13.0 (13.5)	14.0 (14.0)	1.02 (0.99–1.05)
Ever known anyone with TB	210 (41.8)	78 (35.9)	132 (46.2)	1.53 (1.06–2.19)

*Values are no. (%) unless otherwise indicated. IGRA, interferon-γ release assay; CI, confidence interval; IQR, interquartile range; IDUs, injection drug users; TB, tuberculosis.
†Risk groups are not mutually exclusive; reference group includes all participants without the characteristic.
‡Refers to the past 6 mo.

per TB control efforts in the region. Interventions are needed that prevent HIV transmission and LTBI reactivation.

Among IDUs in this study, the TB infection prevalence (63%) was consistent with that in an earlier study of IDUs in Tijuana (*18*), which reported a crude prevalence of 67% and a prevalence of TB infection of 64% after the estimate was adjusted for respondent-driven sampling. This method of sampling of hidden populations enables researchers to adjust prevalence estimates to account for sampling bias (*24*). In addition to confirming the high prevalence of LTBI among IDUs in Tijuana, we found a disturbingly high LTBI prevalence among other hard-to-reach groups. Given that the reference group for associations found between FSWs or homelessness and LTBI status was mostly IDUs, it was not surprising that these factors had odds ratios less than unity. Further studies that include low-risk groups are needed to determine the risk for LTBI among FSWs and homeless persons relative to the general population.

Unlike our earlier study (*18*), in which we referred persons with TB symptoms directly to a community clinic for further evaluation, the current study included AFB smear microscopy and chest radiography to detect active TB before referring participants for care. These modifications enabled us to estimate the prevalence of active TB. Our estimate of TB disease prevalence (398/100,000) was ≈4× higher than the reported TB prevalence for Baja California. Although this estimate is based on a small number of cases, the fact that AFB smears can miss up to 40% of culture-positive TB cases (*25*) indicates that this finding is likely a conservative estimate. Our findings concur with those of other studies, which showed that substance abuse, injection drug use in particular, is associated with increased risk for active TB (*26*).

Age and years of residence in Tijuana were associated with increased odds of TB infection. This finding, along with the fact that TB incidence was estimated to be 57/100,000 in Baja California versus the national rate of 16.2/100,000, suggests that living in this region of Mexico may increase the risk for acquiring *M. tuberculosis* infection (*1*). Although having spent time in jail or prison was associated with TB infection in our study, having spent time in a correctional facility in Mexico appeared to be

a major risk factor, regardless of also having spent time in a correctional facility in the United States. Transmission of TB is facilitated by close contact with infectious persons, such as those found in correctional facilities, and increased TB incidence rates have been documented during incarceration (*27*,*28*). Inmates at facilities in Mexico may have a higher risk for infection because of the higher prevalence of TB in Mexico than in the United States, the greater densities of inmates in these facilities, and the lack of LTBI screening programs in Mexico. These correctional facilities offer a unique opportunity to reduce TB in the community at large (*29*).

The World Health Organization has estimated that one third of the world's population has LTBI (*7*), which creates a massive reservoir of disease that is considered a major threat to global TB control (*30*). Persons co-infected with TB and HIV are at greatest risk for reactivation of LTBI; they have an incidence of ≈35–162 cases/1,000 person-years. The second and third highest risks for LTBI reactivation occurs among persons who were recently infected (<12 months earlier) and IDUs, who collectively have an incidence of ≈10–12.9/1,000 person-years, which is independent of their HIV status (*31*). However, in Mexico sufficient resources are not available to treat all with LTBI cases. Therefore, treatment is restricted to those with LTBI cases considered at high risk for reactivation, which is currently defined as children who have had contact with infectious TB case-patients and those co-infected with HIV. As new diagnostic tools capable of identifying LTBI cases at imminent risk for progressing to TB are developed, TB control programs in places such as Tijuana could expand treatment of persons with LTBI in a focused, cost-efficient manner (*32*).

The cross-sectional design of our study precluded us from drawing temporal inferences between TB infection and risk factors we examined. Moreover, information obtained about recent behavior (substance and drug use, sexual behavior, and housing status) may not reflect patterns present at the time participants became infected. This finding could explain why our analysis identified that only lifetime variables were independently associated with TB infection. In addition, this study used convenience sampling methods to enroll participants. Therefore, our results may not be representative of other marginalized populations in Mexico. *M. bovis* infections, which accounted for ≤17% of TB infections in persons born in Mexico who were living in southern California from 2001 through 2005 (*33*), can also cause an IGRA+ result. Thus, some IGRA+ results in this study may have been caused by *M. bovis* infections rather than *M. tuberculosis* infections. However, we do not believe that this possibility changes our conclusions because TB caused by *M. bovis* and TB caused by *M. tuberculosis* are essentially indistinguishable clinically (*34*), and both infections cause illness and death in this region (*33*), which are exacerbated by HIV infection.

Table 2. Multivariate analysis of factors associated with tuberculosis infection status among high-risk groups for HIV in Tijuana, Mexico, April–July 2007*

Characteristic	Adjusted odds ratio (95% CI)
Age, y	1.03 (1.01–1.05)
Years lived in Tijuana	1.02 (1.01–1.04)
Incarceration status	
Never jailed	1.00
Jailed in USA only	1.61 (0.98–2.63)
Jailed in Mexico or in Mexico and USA	2.28 (1.48–3.51)

*Odds ratios were adjusted for all other variables. CI, confidence interval.

Our results were based on an IGRA, and although there is no standard procedure for detecting LTBI, studies have consistently reported that IGRAs are more sensitive and specific for detecting active TB than are TSTs (*35*). In addition, 78% of the study participants had a visible BCG scar, which suggested that LTBI would have been overestimated if based on a TST in this population. Although the LTBI estimates in this study suggest treatment specific for high-risk groups, additional studies are needed to obtain comparable estimates from the general population to determine whether interventions should be specific for certain groups or the general population.

Although prevention of infection with HIV should be the top priority for reducing TB risk, the high LTBI prevalence found in this study indicates an unmet need for early TB identification and treatment among populations in Tijuana at risk for HIV infection. TB and HIV screening and treatment can be difficult to accomplish, given that these hard-to-reach populations access medical care infrequently, and drug users and homeless populations have additional barriers to compliance with TB treatment regimens (*36*,*37*). Official guidelines in Mexico currently recommend treatment for LTBI only for exposed children <5 years of age, children 5–14 years of age if they have no history or signs of BCG vaccination, and HIV-infected persons with known exposure to a person with an active case of TB (*38*). However, a separate economic analysis has shown that an LTBI screening program similar to that demonstrated in this study, together with an LTBI treatment strategy, would be cost-effective among IDUs in Tijuana (*39*). Additional studies are needed to determine whether treatment of LTBI would also be cost-effective for the general population and other groups at high risk for TB/HIV co-infection. Expanded TB and HIV screening efforts, coupled with HIV risk reduction interventions and education about TB among these high-risk populations, are needed to avoid reactivation and spread of TB resulting from emergence of HIV in this Mexico/United States border region.

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Dr Garfein is an associate professor at the University of California, San Diego. His research interests include TB/HIV coinfection along the Mexico–United States border region and prevention of infected diseases related to substance abuse.

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Anaplasma phagocytophilum from Rodents and Sheep, China

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To characterize the strains of *Anaplasma phagocytophilum* in wild and domestic animals in China, we isolated the organism from rodents and sheep in northeastern China. We isolated 3 strains (2 from rodents and 1 from sick sheep) through propagation in BALB/c mice and then cell culture in HL60 cells. The 3 isolates were identified by Wright-Giemsa staining, immunofluorescence, and electronic microscopy and were characterized by sequence analyses of the 16S rRNA gene, partial citrate synthase gene, major surface protein 4 gene, and heat shock protein gene. The multiple sequences of the 3 isolates were identical to each other but different from all known strains from other countries. The public health and veterinary relevance of the isolates deserves further investigation.

Anaplasma phagocytophilum has been recognized as an animal pathogen and is an emerging human pathogen of public health relevance. From 1994 to 2005, ≈3,000 cases of human granulocytic anaplasmosis were diagnosed in the United States (*1*), and in more recent years, sporadic and clustered cases have been reported in Europe and the People's Republic of China (*2*–*5*). Human are usually infected by tick bites, although perinatal transmission or transmission through contact with infected animal blood has been reported (*1*). A broad variety of animal species are known to carry *A. phagocytophilum,* and humans are incidental dead-end hosts (*6*).

Various *A. phagocytophilum* strains have been isolated from humans (*6*), domestic and wild animals, and ticks in the United States and Europe (*1*,*6*,*7*). Prior serologic and molecular evidence suggests that *A. phagocytophilum* has also infected humans, rodents, and ticks in many Asian countries, including China, Japan, and Korea (*8*–*12*). Our objectives were to obtain isolates of *A. phagocytophilum* in vitro by using the HL60 cell line and to characterize the strains from wild and domestic animals in China.

Materials and Methods

Collection and Preparation of Specimens

In May 2009, live rodents were captured in wire mesh traps in the hinterland of the Changbai Mountains (42°45′N, 130°35′E) in Jilin Province, China, where natural infections with *A. phagocytophilum* in ticks and rodents have been reported (*8*). After their species and sex were identified, trapped rodents were euthanized and anatomized. The spleen was removed from each rodent and ground with sterile normal saline. Four dying sheep were found at the same site at the same time; blood samples were aseptically collected into tubes containing EDTA-K^{2+}.

Propagation of *A. phagocytophilum* in BALB/c Mice

For isolation of *A. phagocytophilum*, the spleen suspensions of the rodents were pooled into 12 groups according to species, and 0.3 mL of spleen suspension was intraperitoneally injected into 48 BALB/c mice (4 in each group). Blood samples from the 4 sheep were also pooled and injected into a group of BALB/c mice by the same means.

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After 7–14 days, blood samples were collected from each inoculated mouse and evaluated for infection by real-time PCR. All animal experiments were performed according to the approved Institutional Animal Care and Use Committee guidelines.

Isolation of *A. phagocytophilum* in HL60 Cells

The HL60 leukemia cell line was used to cultivate *A. phagocytophilum* as described (*13*). A volume of 100–300 μL blood (in EDTA-K^{2+}) from infected BALB/c mice was inoculated into HL60 cells at densities of 2×10^5 to 6×10^5 cells/mL (*13*).

Wright-Giemsa Staining and Immunofluorescence Microscopy

Slides of peripheral blood or the cultured cells were stained with Wright-Giemsa (BaSO DIAGNOSTICS, INC, Zhuhai, China). An indirect immunofluorescence assay was performed after the slides of culture cells were fixed for 10 minutes in a 1:1 solution of methanol and acetone as described (*13*). Horse anti–*A. phagocytophilum* serum (kindly provided by Jenet E. Foley, University of California, Davis, CA, USA) and fluorescein isothiocyanate–conjugated goat antihorse immunoglobulin G (Zhongshan Biotechnique, Inc., Beijing, China) were used for the assay. Serum samples from healthy horses were used as negative controls.

Electronic Microscopy

Infected HL60 cells were processed as previously described (*14*). Electron microscopic examination was conducted by using a Tecnai 10 electron microscope (Philips, Amsterdam, the Netherlands).

PCR and Sequence Analysis

Real-time PCR selective for the major surface protein 2 gene (*msp2*) was used as described by Drazenovich et al. (*15*). To characterize the *A. phagocytophilum* strains isolated in the study, we amplified, purified, sequenced, and compared the 16S rRNA gene (*rrs*), partial sequences of the citrate synthase gene (*gltA)*, major surface protein 4 gene (*msp4*), and heat shock protein gene (*groEL*) as described (*8,16,17*). Phylogenetic analyses were performed and phylogenetic trees were constructed by using Mega 3.0 software (*17,18*).

Nucleotide Sequence Accession Numbers

The nucleotide sequences of *A. phagocytophilum* isolated in this study were deposited in GenBank. Accession numbers are GQ412337–GQ412339 for 1,431-bp *rrs*, GQ412340–GQ412342 for 348-bp to 348-bp *gltA*, GQ412343–GQ412345 for 428-bp *groEL*, and GQ412346–GQ412348 for 779-bp *msp4*.

Results

A. phagocytophilum in BALB/c mice

A total of 47 live rodents—20 black-striped field mice (*Apodemus agrarius*) and 27 great long-tailed hamsters (*Tscherskia triton*)—were captured. When tested 7–14 days postinoculation, every mouse in 5 of the 12 groups of inoculated BALB/c mice was positive for *A. phagocytophilum* according to real-time PCR selective for the *msp2* gene; 3 groups were *A. agrarius* mice and 2 were *T. triton* hamsters. Two BALB/c mice inoculated with the anticoagulated blood samples from the 4 sick sheep were positive for *A. phagocytophilum* according to PCR. Typical morulae were observed in granulocytes of experimentally infected BALB/c mice (Figure 1, panel A).

A. phagocytophilum in HL60 cells

Three *A. phagocytophilum* strains were propagated in HL60 cells: 1 from *A. agrarius* mice was named China-C-Aa, 1 from *T. triton* hamsters was named China-C-Tt, and 1 from sheep was named China-C-Y. *A. phagocytophilum* was first observed in Wright-Giemsa stain preparations 5 days after preparation of cultures (Figure 1, panel B). Morulae were found in ≈70% of HL60 cells at 10 days postinoculation. PCR showed all 3 agents cultured to be *A. phagocytophilum.* Blank control cultures (HL60 cells only) and cultures inoculated with blood of uninfected BALB/c mice showed no evidence of infection by Wright-Giemsa stain or PCR. Immunofluorescence microscopy demonstrated specific staining of *A. phagocytophilum* in infected cells (Figure 1, panel C). Such staining was not observed in uninfected cells or in cells incubated with control serum. Electron microscopy showed cytoplasmic inclusions in infected HL60 cells. The size of individual bacteria varied, and double-layered membranes were clearly observed surrounding electron-lucent and electron-dense forms (Figure 1, panel D).

A. phagocytophilum Isolate Sequences

The 1,431-bp nearly entire *rrs* sequences of the 3 *A. phagocytophilum* isolates from cultured cells were identical to each other and to the sequences amplified from infected mice as well as from field-collected rodents and sheep. The tested *rrs* sequences were also identical to sequences amplified from ticks and rodents captured 3 years ago (GenBank accession nos. DQ342324 and DQ449948) in the same area (*8*) but different from all known *A. phagocytophilum* sequences deposited in GenBank.

Analysis of the partial sequences of *gltA* (348 bp), *msp4* (779 bp), and *groESL* (428 bp) genes showed that the nucleotide sequences of *gltA* fragments amplified from the 3 isolates were identical to each other and showed 84% –99% identity with previously reported *A. phagocytophilum*

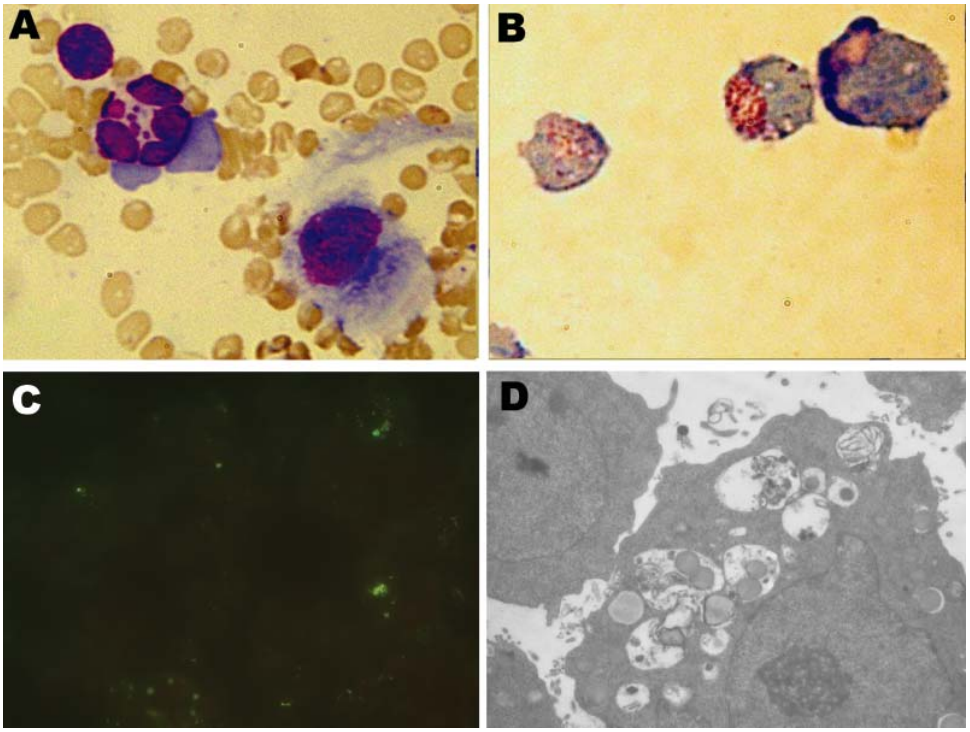

Figure 1. Photomicrographs of cells infected with *Anaplasma phagocytophilum*. A) Wright-Giemsa–stained granulocytic cell of a BALB/c mouse. B) Wright-Giemsa-stained HL60 cells. C) Immunofluorescent-stained infected HL60 cells. D) Electron photomicrographs of an HL60 cell. Original magnifications ×1,500 (A–B), ×1,000 (C) and ×6,200 (D).

strains, with 3–52 bp differences and 83%–99% similarity of deduced amino acid sequences. Three clades were structured on a phylogenetic tree based on 348-bp nt of the *gltA* gene, including a clade of strains from the United States, the Russian Far East, and this study; a clade comprising strains from rodents in southeastern China; and a clade of other *Anaplasma* spp., such as *A. centrale, A. marginale*, and *A. platys* (Figure 2).

The sequences of 779-bp *msp4* fragments amplified from the 3 isolates were also 100% identical and had 98%–87% nt sequence identity and 99%–88% deduced 268-aa sequence identity compared with *A. phagocytophilum* strains available in GenBank. When compared with the sequences from rodents in southeastern China (GenBank accession no. EU008082), nucleotide identity was only 87% with a 95-bp difference, and induced amino acid identity was 88% with a 31-aa difference. Phylogenetic analysis placed the *A. phagocytophilum* isolates in this study on a separate branch and in the same clade as the strains from the United States and Europe but far from the strains from sheep in Norway (GenBank accession no. AY706391), mule deer in Montana (DQ674249), and rodents in southeastern China (EU008082) (Figure 3). The other *Anaplasma* spp. were in a separate clade.

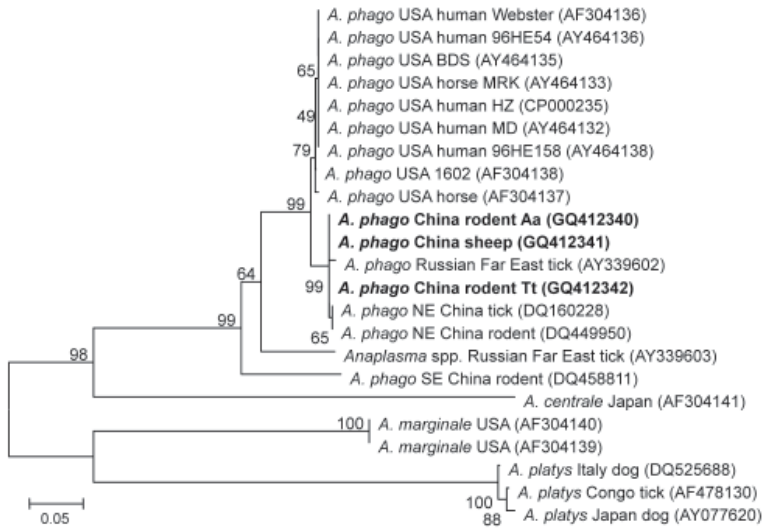

Figure 2. Phylogenetic tree based on partial (348-bp) *gltA* sequences of *Anaplasma* spp., obtained by using neighbor-joining method with Kimura 2-parameter analysis and bootstrap analysis of 1,000 replicates. Numbers on the branches indicate percentage of replicates that reproduced the topology for each clade. Parentheses enclose GenBank numbers of the sequences used in the phylogenetic analysis. **Boldface** indicates sequences obtained from rodents and sheep from northeastern China, May 2009. Scale bar indicates number of nucleotides per 1,000 bp. *phago, phagocytophilum.*

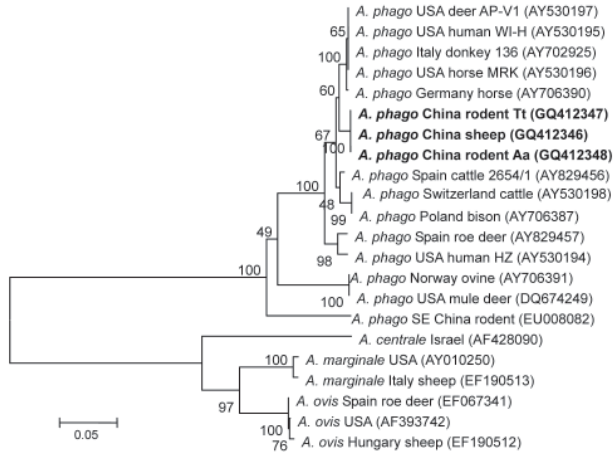

Figure 3. Phylogenetic tree based on partial (779-bp) *msp4* nt sequences of *Anaplasma* spp., obtained by using the neighbor-joining method with Kimura 2-parameter analysis and bootstrap analysis of 1,000 replicates. Numbers on branches indicate percent of replicates that reproduced the topology for each clade. Parentheses enclose GenBank numbers of the sequences used in the phylogenetic analysis. **Boldface** indicates sequences obtained from rodents and sheep from northeastern China, May 2009. Scale bar indicates number of nucleotides per 1,000 bp. *phago, phagocytophilum.*

When the 428-bp *groEL* sequences of the 3 isolates were compared with known *A. phagocytophilum* sequences in GenBank, the identity varied from 93% to 99%. The phylogenetic tree of *groEL* showed the *A. phagocytophilum* isolates in this study on a separate branch. The strains from humans in China and the United States, horses in United States, dogs in Slovenia, roe deer in Poland and Austria, and ticks in Germany were in another clade (Figure 4); however, their deduced amino acid sequences were identical to those from patients and rodents in southeastern China.

Discussion

We isolated 3 strains of *A. phagocytophilum* from black-striped field mice, great long-tailed hamsters, and sheep in northeastern China. The availability of the isolates in a cell line will permit studies on the genetic, proteomic, and pathogenic characteristics of this agent.

A. phagocytophilum is reportedly maintained in various animal reservoirs, such as white-footed mice (*19*), woodrats (*7*), goats, sheep, and horses (*5*,*20*). Our isolation of 3 *A. phagocytophilum* strains from *A. agrarius* and *T. triton* rodents and from sheep indicates that both small wild animals and domestic animals may act as competent reservoirs of *A. phagocytophilum* in northeastern China. Although we found cultivation of this organism from experimentally infected mice to be reliable, the sensitivity of cultivation from wild and domestic animals is uncertain. In addition, the specimens used for isolation were pooled. Consequently, we were unable to ascertain the exact prevalence of infection in the rodents collected for this study. In a previous survey, we found a natural infection rate of 8.8% for *A. phagocytophilum* in rodents in the same area (*8*). To determine the level of infectivity in rodents as well as domestic animals, further studies are needed.

The nucleotide sequences of the 3 strains in this study were identical to each other in corresponding genes. The 1,431-bp nearly entire *rrs* sequences were most closely related to those detected in rodents from southeastern China (*8*,*9*), but they differed from other known strains. The sequence divergences and the phylogenetic analyses of partial *gltA*, *msp4*, and *groESL* genes indicated that a novel strain of *A. phagocytophilum* might be prevalent in northeastern China.

Different *A. phagocytophilum* strains seem to have special host tropisms (*21*). Strains from sciurids and white-footed mice infect various laboratory animals and perhaps humans as well. *A. phagocytophilum*–variant 1 and the strains from woodrats are found in association with wildlife only; human infections with these strains have yet to be identified. *A. phagocytophilum*–variant 1 has been unable to infect white-footed mice or SCID (severe combined immunodeficiency) mice but could infect goats by experimental inoculation (*22*). Holden et al. have documented that

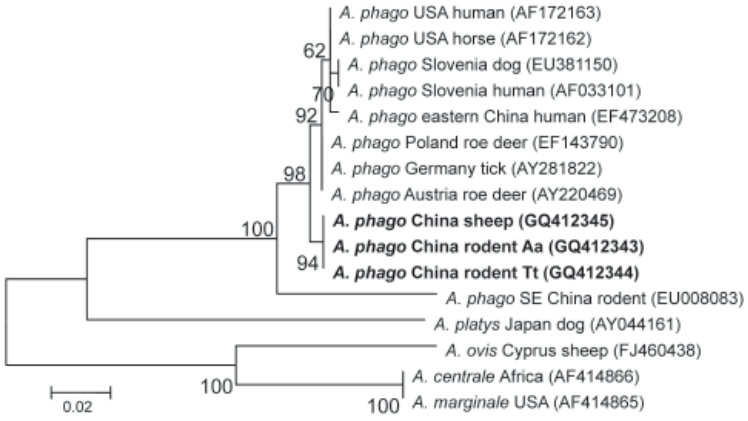

Figure 4. Phylogenetic tree based on partial (428-bp) *groEL* nt sequences of *Anaplasma* spp., obtained by using the neighbor-joining method with Kimura 2-parameter analysis and bootstrap analysis of 1,000 replicates. Numbers on branches indicate percent of replicates that reproduced the topology for each clade. Parentheses enclose GenBank numbers of the sequences used in the phylogenetic analysis. **Boldface** indicates sequences obtained from rodents and sheep from northeastern China, May 2009. Scale bar indicates number of nucleotides per 1,000 bp. *phago, phagocytophilum.*

the pathogenicity of an *A. phagocytophilum* strain causing human disease waned with mouse passage in C3H mice but could be resurrected by passage in SCID mice (*23*). In our study, *A. phagocytophilum* strains with the same molecular characteristics were isolated not only from wild rodents but also from domestic sheep. Furthermore, they could propagate in BALB/c mice in the laboratory. The host tropisms and pathogenicity of the isolates remain to be clarified, and the relevance of these findings to public health and veterinary medicine deserves further investigation.

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Spread of Adenovirus to Geographically Dispersed Military Installations, May–October 2007

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In mid-May 2007, a respiratory disease outbreak associated with adenovirus, serotype B14 (Ad14), was recognized at a large military basic training facility in Texas. The affected population was highly mobile; after the 6-week basic training course, trainees immediately dispersed to advanced training sites worldwide. Accordingly, enhanced surveillance and control efforts were instituted at sites receiving the most trainees. Specimens from patients with pneumonia or febrile respiratory illness were tested for respiratory pathogens by using cultures and reverse transcription–PCR. During May through October 2007, a total of 959 specimens were collected from 21 sites; 43.1% were adenovirus positive; the Ad14 serotype accounted for 95.3% of adenovirus isolates. Ad14 was identified at 8 sites in California, Florida, Mississippi, Texas, and South Korea. Ad14 spread readily to secondary sites after the initial outbreak. Military and civilian planners must consider how best to control the spread of infectious respiratory diseases in highly mobile populations traveling between diverse geographic locations.

Adenovirus (Ad)–associated acute respiratory disease (AdARD) epidemics have been widely reported among recruits at US Department of Defense (DoD) training centers (*1*–*5*). Vaccines targeting Ad4 and Ad7, the most common serotypes associated with these illnesses, were used among United States military trainees from 1971 through early 1999, when the supply was exhausted following cessation of vaccine production in 1996 (*1*). Because of the historically high negative effects of respiratory disease and the discontinuation of vaccine, the DoD initiated a population-based, active surveillance program in 1996 to track ARD activity among recruits at 8 military training centers, including the Air Force's only recruit training center at Lackland Air Force Base (AFB) in San Antonio, Texas (*1*,*6*,*7*).

Lackland AFB admits 400–800 new basic military trainees (BMTs) per week; ≈35,000 BMTs graduate annually. BMTs are assigned to flights of 45–65 persons during the 6.5-week training program. All flight members train, eat, and sleep as a unit and are housed in 1 large open-bay facility. According to DoD surveillance data, during January 2005–January 2007 Lackland AFB experienced relatively mild ARD activity among BMTs; rates ranged from 0.1–0.7 cases per 100 recruit-weeks (US Naval Health Research Center, unpub. data). No adenovirus-positive specimens from Lackland AFB were serotyped during 2005, and only 4 were serotyped during 2006; serotypes included 1 Ad21, 1 AdC, and 1 Ad3. One specimen showed an Ad14/Ad21 co-infection (*8*). Adenovirus serotype B14 (Ad14) was detected at Lackland AFB for the first time in 2006; in that same year, Ad14 was also detected at 3 other DoD training centers (*8*).

Beginning in February 2007, an outbreak of respiratory illness associated with Ad14 occurred among Lackland AFB BMTs. During the height of the outbreak in June 2007, ARD rates exceeded 2.0 cases per 100 recruit-weeks

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(Naval Health Research Center, unpub. data). Most cases involved only mild, acute, febrile, respiratory illness. However, during April–October 2007, 27 patients were hospitalized with pneumonia and more severe sequelae; some patients required intensive care. All these patients were found to be adenovirus positive, and 20 (74.1%) had positive tests for the Ad14 subtype. The recognition of these more severe cases prompted an investigation and enhanced surveillance to describe the clinical and epidemiologic characteristics of Ad14 in this population. Laboratory results from early in the investigation indicated that 63% of ARD-related respiratory specimens collected from BMTs were positive for adenovirus and that 90% of adenovirus infections were the Ad14 subtype (*9*). Most BMTs became ill with adenovirus in training weeks 4 and 5 (US Naval Health Research Center, unpub. data) and may have still been infectious after graduation because virus shedding can occur in respiratory secretions and feces for several weeks (*10–12*).

We modeled the transmission of Ad14 through 2 hypothetical flights containing 50 BMTs each (Figure 1) by using data based on actual laboratory results and epidemiologic findings from Lackland AFB; our model indicated that >50% of BMTs, during the height of the outbreak, were infected with Ad14 over the course of the 6.5 week training period (*9;* Naval Health Research Center, unpub. data). At the end of basic training, with the conservative assumption that recovering patients shed virus for up to 1 month, ≈28% of BMTs were still infectious at graduation and in the following days or weeks. Given the likelihood that some BMTs were still ill or shedding Ad14 after completing basic training, response and control efforts had to account for the high mobility of this population.

Following graduation, students immediately dispersed to >130 secondary DoD sites for advanced training (Figure 2); most went to a few large Air Force training centers in the United States, while a few went to smaller sites worldwide. Secondary training sites, including Sheppard AFB (Wichita Falls, TX, USA), Goodfellow AFB (San Angelo, TX, USA), and Keesler AFB (Biloxi, MS, USA), began reporting increased ARD among their trainees in mid to late May 2007. We report the spread of Ad14 to secondary training installations and subsequent response efforts, following the Lackland AFB outbreak, from May 25 through October 31, 2007.

Methods

Surveillance

In late May 2007, enhanced, active ARD surveillance was initiated at 12 military installations that received basic training graduates, including 5 Air Force secondary training sites that received the most graduates from Lackland AFB: Sheppard AFB, (28.4% of BMT graduates); Keesler AFB, (16.4%); Goodfellow AFB, (3.8%); Hurlburt Field, Florida (1.8%); and Brooks City-Base, Texas (0.7%). In total, the 12 sites participating in enhanced surveillance efforts received 54.2% of BMT graduates moving to non–Lackland AFB sites for their secondary training.

Staff from the US Air Force School of Aerospace Medicine (USAFSAM) sent respiratory specimen collection kits and educational materials to the secondary sites that had cases, created a website to disseminate information, and encouraged participation through regular email correspondence. Investigators at USAFSAM enhanced surveillance efforts by directing efforts to sites with suspected cases but decreasing or few specimen submissions.

Nasal wash, nasopharyngeal swab, and oropharyngeal (OP) swab specimens were collected from patients meeting the ARD case definition May 25–October 31, 2007, and sent to USAFSAM in viral Universal Transport Medium (Copan, Brescia, Italy). The ARD case definition included fever of ≥100.5°F with a cough or sore throat or evidence of pneumonia. Routine patient surveys that accompanied laboratory specimens were reviewed to obtain patients' demographic data, signs and symptoms, additional clinical information, travel history, and lost training days.

Additionally, staff from USAFSAM and the Air Education and Training Command headquarters coordinated the public health response and provided guidance on prevention, enhanced surveillance, and control efforts. Sites not included in initial enhanced surveillance efforts were also invited to send specimens collected from patients whose conditions met the case definition. By using laboratory surveillance data, weekly ARD and AdARD trends were tracked at the 3 main sites reporting increased ARD, Sheppard AFB, Keesler AFB, and Goodfellow AFB.

Laboratory Methods

Specimens were tested by traditional viral culture,

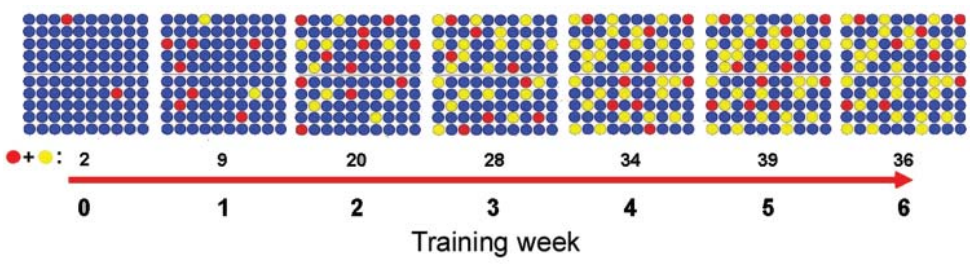

Figure 1. Evolving adenovirus subtype B14 incidence rate per 100 US Air Force basic military trainees over 6.5 weeks of basic training, based on epidemiologic and laboratory surveillance data. Red circles, acutely ill; yellow circles, recovering/possibly infectious; blue circles, well.

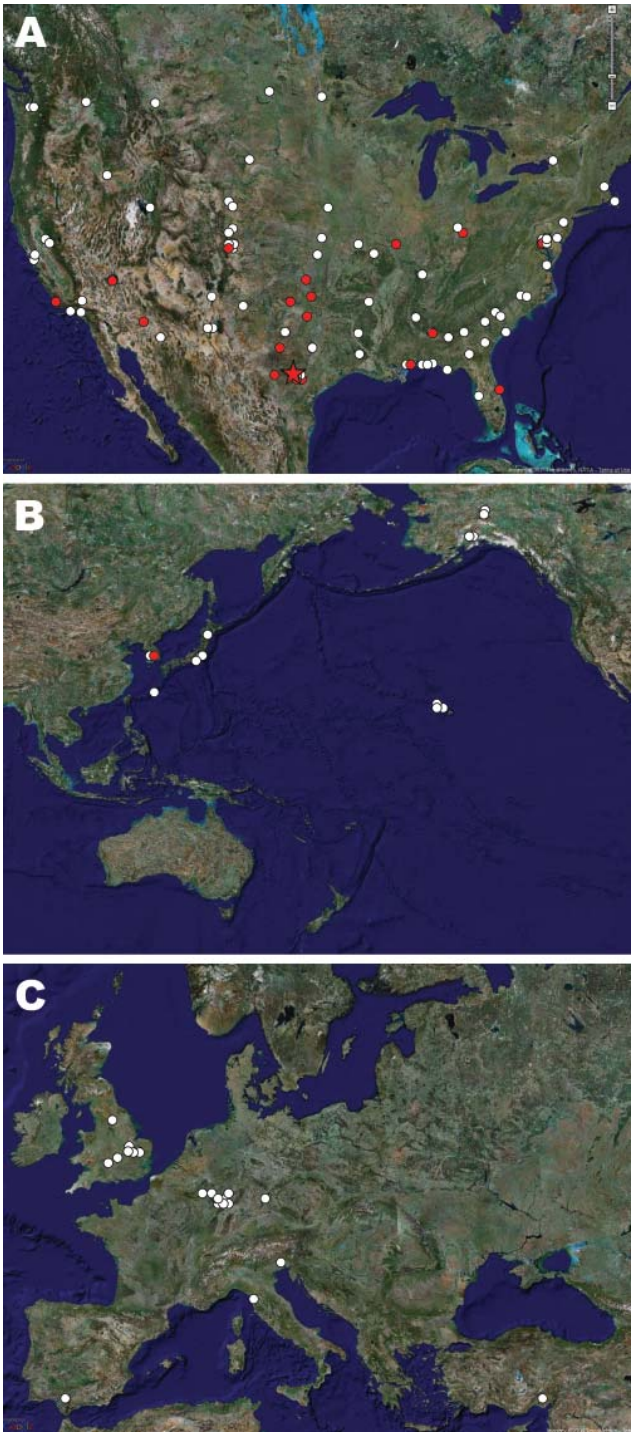

Figure 2. Locations of military sites that received US Air Force basic military training graduates for secondary training in North America (A), the Pacific region (B), and Europe and the Middle East (C). Red indicates locations that submitted specimens as part of adenovirus surveillance. Star in panel A indicates Lackland Air Force Base, Texas, USA. Maps generated by using TerraMetrics (www.terrametrics.com).

shell vial culture (R-Mix; Diagnostic Hybrids, Athens, OH, USA), and, beginning July 25, 2007, reverse transcription–PCR (RT-PCR) for subtype B14. Most adenovirus culture–positive specimens submitted between May 26 and July 25, 2007 were tested for Ad14 after the test capability became available. Viral and shell vial culture identified adenovirus, influenza, parainfluenza viruses 1–3, respiratory syncytial virus, and rhinovirus, as well as herpes simplex virus 1 and enterovirus after additional evaluation. Tube cultures were examined for 10 days for cytopathologic effects, and cells from the shell vial cultures were stained with pooled fluorescent antibodies and virus-specific monoclonal antibodies. The procedure and hexon-specific oligonucleotides for the adenovirus B14-specific monoplex RT-PCR were adapted from a US Naval Health Research Center protocol (*8*,*13*). On nasal wash and OP swab specimens and adenovirus isolates, DNA was extracted from the transport media and amplified by RT-PCR. The resulting RT-PCR products were then purified by using Millipore (Billerica, MA, USA) microcolumns and subsequently analyzed by agarose gel electrophoresis, DNA sequencing, or both. The analyte-specific reagent (ASR) primers and laboratory-developed diagnostic assay were used in accordance with requirements specified by the College of American Pathologists for use as part of molecular diagnostics testing performed at USAFSAM.

Prevention and Control

In addition to adopting prevention and control measures to mitigate transmission within its own training population (*9*), Lackland AFB officials initiated actions to reduce spread to secondary training sites. Personnel screened outgoing BMTs from Lackland AFB for fever by placing chemical temperature dots on the forehead. Students with a temperature ≥100.5°F were held back from travel and housed in a medical-hold dormitory until their measured temperatures dropped below 100.5°F for 24 hours. Secondary training sites also adopted prevention and control measures to help incoming students and other assigned active duty members remain as healthy as possible.

Because it typically receives the most BMT graduates, Sheppard AFB instituted more aggressive case-finding procedures and prevention measures than any other secondary training site and fully implemented these actions by June 8, 2007, 12 days after enhanced surveillance efforts began. Their prevention efforts are described here; several other secondary sites instituted similar practices. All students arriving from Lackland AFB were screened for a measured fever ≥100.5°F and administered a questionnaire during in-processing. Students suspected of having ARD were further screened by a healthcare provider and sent to the clinic for treatment and testing as appropriate. Students with ARD were issued masks, grouped with other ARD stu-

dents, placed on quarters (confined to their living area and restricted from participating in all work and leisure activities), and removed from all training activities. Students on quarters were not allowed to enter dining halls, and meals were instead brought to their rooms. Students were reevaluated by a healthcare provider after 24 hours on quarters and returned to duty if afebrile.

Sheppard AFB mandated that a virucidal cleaning agent be used several times per day to sanitize high-touch surfaces in facilities, including dining halls, classrooms, dormitories, buses, taxis, the post office, and other student-frequented establishments. In addition, hand washing and use of hand sanitizer were highly encouraged and closely monitored.

Upon completion of the training program, outgoing students were also screened for ARD by using the same questionnaire and a documented temperature. All students suspected of having ARD were placed on medical hold and evaluated by a physician to determine whether treatment was needed. After 24 hours they were reevaluated and released to travel if afebrile.

Results

From May 25 through October 31, 2007, USAFSAM received 959 respiratory specimens from the 12 secondary training sites that initially participated in enhanced surveillance and from 9 additional sites (Table 1). Adenovirus accounted for 413 (89.8%) of the 460 specimens with known etiologic agents; the other viruses identified included parainfluenza (31 [6.7%]), influenza type A (5 [1.1%]), respiratory syncytial virus (2 [0.4%]), and enterovirus (1 [0.2%]). Among the specimens that were culture positive for adenovirus, 358 (86.7%) were tested for Ad14, of which 341 (95.3%) were positive. Ad14 was identified at 8 secondary sites located in California, Florida, Mississippi, Texas, and South Korea; collection dates of the first Ad14-positive specimen at each site ranged from May 30 through October 30. Most patients (331 [97.1%]) with confirmed Ad14 infection were advanced training students, while 9 (2.6%) infections occurred in active duty members outside the training population, and 1 (0.03%) occurred in a dependent child.

Patient survey data were available for 538 of the 959 (56.1%) patients from whom specimens were collected; of these, 220 (40.9%) were Ad14 positive. The following results are only for those 220 patients with confirmed Ad14 infection and available patient survey data (Table 2). Patient ages spanned 17–29 years, though most (183 [84.7%]) patients were 18–22 years of age; the median age was 19 years. In addition, most (197 [89.5%]) patients were male. Regarding patient symptoms, the median temperature recorded was 101.0°F. The most common signs and symptoms reported by patients were sore throat (90.9%), chills (83.2%), fatigue (78.6%), cough (78.2%), headache (75.9%), body aches (70.0%), and nasal congestion (61.4%). One patient was hospitalized with pneumonia

Table 1. Summary of results from respiratory specimens received from USAF secondary training bases, May 25–October 31, 2007*

Site†	No. specimens	Adenovirus not otherwise specified, no. (%)	Ad14, no. (%)	Date first Ad14-positive specimen collected
Altus AFB, OK	2	0	0	–
Andrews AFB, MD	12	1 (8)	0	–
Bolling AFB, DC	1	0	0	–
Brooks City-Base, TX	10	1 (10)	1 (100)	Jun 30
Goodfellow AFB, TX	71	37 (52)	19 (51)	Jun 1
Hurlburt Field, FL	3	2 (67)	2 (100)	Oct 22
Keesler AFB, MS	85	46 (54)	38 (83)	May 31
Laughlin AFB, TX	11	2 (18)	0	–
Luke AFB, AZ	3	1 (33)	0	–
Maxwell AFB, AL	23	1 (4)	0	–
Nellis AFB, NV	3	0	0	–
Osan AB, South Korea	6	1 (17)	1 (100)	Jun 19
Patrick AFB, FL	2	0	0	–
Randolph AFB, TX	9	1 (11)	1 (100)	Oct 30
Scott AFB, IL	9	2 (22)	0	–
Sheppard AFB, TX	683	309 (45)	273 (88)	May 30
Tinker AFB, OK	6	2 (33)	0	–
USAF Academy, CO	6	0	0	–
Vance AFB, OK	0	0	0	–
Vandenberg AFB, CA	10	7 (70)	6 (86)	Jun 14
Wright-Patterson AFB, OH	4	0	0	–
Total	959	413 (43)	341 (83)	

*USAF, United States Air Force; Ad14, adenovirus B14; AFB, Air Force Base; OK, Oklahoma; MD, Maryland; DC, District of Columbia; TX, Texas; FL, Florida; MS, Mississippi; AZ, Arizona; AL, Alabama; NV, Nevada; AB, Air Base; IL, Illinois; CO, Colorado; CA, California; OH, Ohio.
†All sites located in the United States except Osan AB (South Korea).

and recovered fully without complications. A total of 191 (86.8%) patients were placed on quarters. Of the 125 patients for whom length of quarters information was available, most (108 [86.4%]) were placed on quarters for 24 hours. In addition, 147 (66.8%) patients had recently traveled; of these, most (143 [97.3%]) had recently traveled from Lackland AFB.

At the 3 secondary sites receiving the most BMT graduates, Sheppard AFB, Goodfellow AFB, and Keesler AFB, AdARD incidence rates among active duty personnel were tracked and compared with concurrent rates calculated at Lackland AFB (Figure 3). AdARD rates at Lackland AFB ranged from 0.1–2.0 cases per 100 personnel, with 2 peaks in June and September 2007. AdARD activity at Sheppard AFB waxed and waned throughout the surveillance period, ranging from 0.2–0.8 ARD cases per 100 personnel. The largest peak of activity occurred on September 22, 2007, 2 weeks following the onset of Lackland AFB's second wave of activity. However, this AdARD activity was short lived, decreasing over a course of 4 weeks to 0.2 cases per 100 personnel. Activity at Goodfellow AFB and Keesler AFB was highest following the initial peak at Lackland AFB, and then tapered off. All 3 sites placed ill students on quarters, which resulted in the short-term removal of >600 students from training activities. Only 1 person required hospitalization for adenovirus-associated pneumonia, at Sheppard AFB, during this time (0.01/100 trainees for this 23-week time period). As of October 31, 2007, prevention and control efforts were terminated at Goodfellow AFB and Keesler AFB but continued at Sheppard AFB.

Table 2. Demographic data, symptoms, and other information collected from 220 patients with positive test results for adenovirus serotype B14, May 25–October 31, 2007*

Parameter	Value
Median age, y (range), n = 216	19 (17–29)
Gender	
F	23 (10.5)
M	197 (89.5)
Base where stationed	
Goodfellow AFB, TX	8 (3.6)
Hurlburt Field, FL	2 (0.9)
Keesler AFB, MS	16 (7.3)
Sheppard AFB, TX	188 (85.5)
Vandenberg AFB, CA	6 (2.7)
Signs and symptoms	
Body aches	154 (70.0)
Chest pain	38 (17.3)
Chills	183 (83.2)
Conjunctivitis	24 (10.9)
Cough	172 (78.2)
Diarrhea	42 (19.1)
Dyspnea	36 (16.4)
Earache	60 (27.3)
Fatigue	173 (78.6)
Headache	167 (75.9)
Runny nose	93 (42.3)
Sinus congestion	135 (61.4)
Sore throat	200 (90.9)
Stiffness	89 (40.5)
Vomiting	39 (17.7)
Median clinical temperature	101°F
Placed on quarters	191 (86.8)
Time on quarters, n = 125	
24 h	108 (86.4)
48 h	13 (10.4)
72 h	4 (3.2)
Hospitalized	1 (0.5)
Received influenza vaccine	68 (30.9)
Recent travel	147 (66.8)
Recent travel locations, n = 147	
Lackland AFB, TX	143 (97.3)
Keesler AFB, MS	2 (1.3)
Albuquerque, NM	1 (0.7)
Panama City, FL	1 (0.7)

*Values are no. (%) except as specified. Complete information not available for all patients; n values given when below 220. All locations in United States. AFB, Air Force Base; TX, Texas; FL, Florida; MS, Mississippi; CA, California; NM, New Mexico.

Discussion

Ad14 spread readily to secondary training sites because of the rapid mobility of BMTs following their graduation from basic training. For the most part, the onset of Ad14-related illness occurred first at sites that received the most BMT graduates. Although Lackland AFB made a concerted effort to identify and segregate outgoing febrile BMTs, more than one quarter of the trainees were likely shedding virus or recovering from illness. In addition, many were possibly preclinical and incubating adenovirus as they departed Lackland AFB, with illness developing shortly after arrival at secondary sites.

Although Ad14 was exported continuously to the secondary sites, neither the ARD rates nor the severity of illness at those sites reached the levels seen at Lackland AFB. Control efforts by Lackland AFB that placed febrile BMTs on medical hold and prevented them from leaving the base seemed to affect severity of illness at Lackland AFB and likely reduced the number of ill persons arriving at secondary sites. The lower rates of illness at secondary sites may also have been due to a decreased number of susceptible persons in the secondary training population, berthing differences that resulted in less contact between trainees at secondary sites, and decreased stress levels among trainees. Additionally, decreased illness severity at the secondary training sites may have resulted from the early identification of patients with suspected cases and their placement on quarters, allowing for rest and recovery. Illness trends at Sheppard AFB tracked Lackland AFB ARD activity most closely, possibly because Sheppard AFB received the largest proportion of BMT graduates compared with other secondary training sites and because healthcare personnel

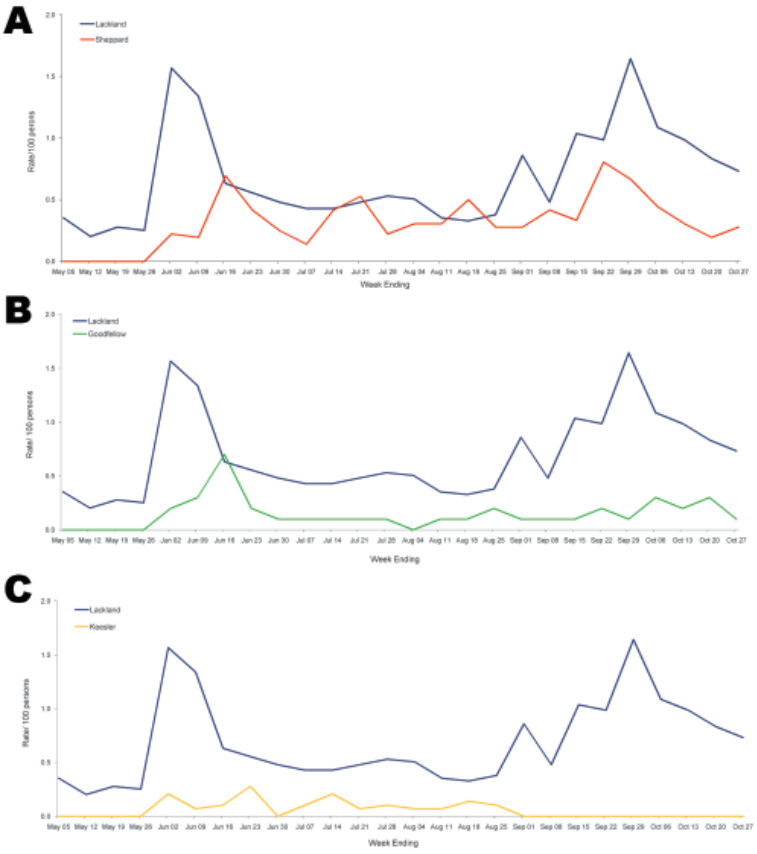

Figure 3. Rates of confirmed adenovirus infection for secondary training students at Sheppard Air Force Base, Texas, USA (A); Goodfellow Air Force Base, Texas, USA (B); and Keesler Air Force Base, Mississippi, USA (C), compared with rates for basic military trainees at Lackland Air Force Base, Texas, USA, May 25–October 31, 2007.

at that base more aggressively identified cases and submitted specimens. Keesler AFB and Goodfellow AFB experienced an earlier decline of infection rates due partially to a lower influx of BMTs from Lackland and partially due to lessening participation in enhanced surveillance efforts after the first 3–4 weeks of surveillance. Adenovirus rates mirrored the overall ARD rates, which suggests that adenovirus accounted for most of the ARD cases during the entire period. As of October 31, 2007, ARD rates had subsided somewhat but not sufficiently to cease surveillance and control efforts.

Surveillance findings indicated that spread of Ad14 to active duty members and dependents outside the training population was minimal, although surveillance efforts were not as robust in these populations. Because secondary training students are segregated from the base population at installations, limited contact takes place between trainees and other military members and their dependents. Still, the lack of spread of this readily transmissible pathogen to persons outside the training population was remarkable.

Eliminating the spread from Lackland AFB to secondary training sites was difficult, because any control measures deployed had to operate within the constraints of the recruit training system. Particularly in wartime, military operational requirements do not permit slowing or canceling the training of new military recruits. Even within this limited setting, segregating ill patients and checking students for fever both before leaving Lackland AFB and upon arrival at their next duty station seemed to affect transmission, because ARD rates remained relatively low at secondary training sites and peak ARD activity did not persist.

A new vaccine for Ad4 and Ad7 is currently under development; phase III has been completed (*14*). The degree of cross-protection this vaccine will offer against Ad14 is unknown, although evidence suggests some protection can be expected. Findings from the Lackland AFB outbreak indicated that Ad7 serum neutralizing antibody was protective against Ad14 illness, mitigating the severity of symptoms (*12*). Previous studies have indicated that Ad4 and Ad7 vaccination was also protective against Ad3 (*8*,*15*) and Ad14 (*8*,*15*–*18*). Thus, implementing the Ad4 and Ad7 vaccine may affect AdARD rates in general in future military trainees.

Following the loss of the Ad4 and Ad7 vaccines, the spread of Ad4 from an Army basic training site to a secondary training installation was associated with a large Ad4 outbreak at a secondary site (*19*). In this report we describe the spread of Ad14 to Air Force secondary training sites by recently graduated basic trainees who moved

quickly from Lackland AFB to their next assignment. Military planners must focus on how best to control the spread of infectious respiratory diseases in highly mobile military populations that travel between geographically dispersed locations. Additionally, these planners must consider that rigid public health interventions may be unacceptable because of interference with critical operations. In this instance, we found that interventions could not interfere with the flow of programmed training operations for a military at war. Additionally, detailed studies aimed at better describing the transmission of adenoviruses may result in better focused and more effective control measures. Our results show that public health leaders in both the military and civilian communities should be concerned about the geographic spread of respiratory disease agents by highly mobile populations.

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At the time this study was conducted, Ms Trei was an epidemiologist at the United States Air Force School of Aerospace Engineering in San Antonio, Texas. Her research interests include infectious disease epidemiology with a focus on respiratory and sexually transmitted diseases.

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Tropheryma whipplei in Children with Gastroenteritis

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Learning Objectives

Upon completion of this activity, participants will be able to:

- Describe the epidemiology of *Tropheryma whipplei* gastroenteritis
- Identify the bacterial load associated with *T. whipplei* gastroenteritis
- Specify laboratory findings associated with *T. whipplei* gastroenteritis
- Compare clinical findings of *T. whipplei* gastroenteritis with other infectious gastroenteritis.

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Tropheryma whipplei, which causes Whipple disease, is found in human feces and may cause gastroenteritis. To show that *T. whipplei* causes gastroenteritis, PCRs for *T. whipplei* were conducted with feces from children 2–4 years of age. Western blotting was performed for samples from children with diarrhea who had positive or negative results for *T. whipplei*. *T. whipplei* was found in samples from 36 (15%) of 241 children with gastroenteritis and associated with other diarrheal pathogens in 13 (33%) of 36. No positive specimen was detected for controls of the same age (0/47; p = 0.008). Bacterial loads in case-patients were as high as those in patients with Whipple disease and significantly higher than those in adult asymptomatic carriers (p = 0.002). High incidence in patients and evidence of clonal circulation suggests that some cases of gastroenteritis are caused or exacerbated by *T. whipplei*, which may be co-transmitted with other intestinal pathogens.

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For decades, Whipple disease was considered to be a metabolic disorder in humans (*1*). An accumulation of data, such as antimicrobial drug susceptibilities and observation of atypical bacteria in intestinal macrophages, has suggested that this disease is an infectious disease. *Tropheryma whipplei* is recognized as the infectious agent responsible for Whipple disease (*2*). Recent studies using molecular biology (*3*–*5*) and culture-dependent (*6*–*10*) techniques have enabled the *T. whipplei* genome to be fully sequenced (*11*,*12*) and have resulted in development of new culture media (*13*), selection of highly sensitive primers for quantitative PCR (*14*), and genotyping (*15*–*19*). The bacterium is found in a viable form in stools of infected patients (*9*).

Until recently, *T. whipplei* was considered a rare bacterium that caused an uncommon disease (*2*). However, recent studies have confirmed that *T. whipplei* is common in stool samples (*20*,*21*). *T. whipplei* DNA has been detected in sewage and is highly prevalent in the feces of sewer workers (12%–26%) (*1*,*20*,*22*–*25*). Moreover, the prevalence of *T. whipplei* in feces of healthy children 2–10 years of age who lived in rural Senegal (sub-Saharan Africa) was 44% (46/105) (*21*). These data, together with the genetic heterogeneity of *T. whipplei* (*19*), indicate that *T. whipplei* is a rather common gut bacterium.

We hypothesize that *T. whipplei* may cause gastroenteritis in children as a result of its primary contact with humans. In a preliminary study, we tested all patients with diarrhea at the University Hospitals in Marseille, France, and determined that this bacterium was found most often in children 2–4 years of age (*26*).

To investigate whether *T. whipplei* caused gastroenteritis, we studied the prevalence of *T. whipplei* DNA in a prospective study of children with diarrhea and controls with diarrhea. We genotyped *T. whipplei* to identify circulating clones (*9*) and report overall findings, including data from the preliminary study.

Materials and Methods

Patients

The study was reviewed and approved by the local ethics committee (agreement no. 07–006). A case-patient was defined as a child 2–4 years of age who had 2 positive quantitative PCR results specific for 2 *T. whipplei* DNA sequences, as reported (*14*,*20*). From January 2006 through December 2008, we tested 241 stool samples from 241 children with diarrhea at 2 University Hospitals for children in Marseille, France (Timone and Nord Hospitals) using a *T. whipplei*–specific PCR. Samples were obtained from all children in accordance with routine hospital procedures. All stools samples were handled identically. Among 36 children with gastroenteritis and *T. whipplei* DNA in stools, 11 stool specimens from 10 children were obtained after their recovery from diarrheal illness.

Five stool specimens were tested ≈15 days later, and 6 were sampled ≈1 month later. Eight serum samples from children with gastroenteritis and positive PCR results for *T. whipplei* were tested retrospectively for *T. whipplei* by Western blot analysis. Data for patients with a definite diagnosis of Whipple disease and positive PCR results for *T. whipplei* PCR in stools at the time of diagnosis and data for adult asymptomatic carriers of *T. whipplei* in stools, which have been reported (*14*,*20*), were also included for quantitative comparisons.

Controls

All *T. whipplei*–infected case-patients were compared with 67 gastroenteritis case-patients of the same age. These 67 children had negative results for *T. whipplei* in stool specimens; epidemiologic, clinical, and biologic features were available for these children.

Forty-seven stool specimens from children 2–4 years of age without gastroenteritis were also tested for *T. whipplei* by using PCR. Twenty-five children (11 girls and 14 boys) were from kindergarten classrooms at the university hospitals. Samples were also obtained from 10 children (4 boys and 6 girls) hospitalized at Timone Hospital for surgery and from 12 children (5 boys and 7 girls) who visited the Emergency Department of Nord Hospital.

Twenty-five serum samples obtained from children 2–4 year of age with gastroenteritis and *T. whipplei*–negative results in stool specimens were tested retrospectively. Twenty control serum samples were obtained from 20 children 1–36 months of age with a disease other than gastroenteritis. All serum samples were from children hospitalized in the 2 University Hospitals. These samples were obtained for routine management of these patients, not specifically for our study.

Diagnostic Procedures

Bacteria, viruses, and *Giardia duodenalis* were detected by using standard methods. Stool specimens were plated onto Hektoen, Campylosel, and *Yersinia* cefsulodin–irgasan–novobiocin agar plates (bioMérieux, Marcy L'Etoile, France). Plates selective for *Campylobacter* spp. were incubated under microaerophilic conditions; all other media and samples were incubated in ambient air. Temperature of incubation was 37°C, with the exception of *Yersinia* agar, which was incubated at 30°C. Length of incubation was 5 days. For virus tests, stool specimens were tested by using a chromatographic immunoassay with a VIKIA Rota-Adeno Kit (bioMérieux) kit and electron microscopy with negative staining, which enabled detection of rotavirus, adenovirus, calicivirus, astrovirus, Norwalk virus, coronavirus, and enterovirus. *G. duodenalis* was detected by PCR, as described (*27*).

T. whipplei quantitative PCR assays of stool samples were performed as reported (*14*,*20*,*28*). Approximately 1 g of stool was obtained for DNA extraction by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), which was performed according to the manufacturer's recommendations. The first *T. whipplei*–specific quantitative PCR specific for a 155-bp sequence used primer pair TW27 forward (5′-TGTTTTGTACTGCTTGTAACAGGATCT-3′) and TW182 reverse (5′-TCCTGCTCTATCCCTCCTAT CATC-3′) and a Taqman probe (27 forward–182 reverse, 5′-6-FAM-AGAGATACATTTGTGTTAGTTGTTACA -TAMRA-3′). The PCR was conducted in a LightCycler

(Roche Diagnostics, Meylan, France) (*14*,*20*,*28*) in a final volume of 20 μL that contained 10 μL of the probe, master kit (QIAGEN), 0.5 μL (10 pmol/μL) of each primer, 5 μL (2 μmol/μL) of probe, 2 μL of distilled water, and 2 μL of extracted DNA. The amplification conditions involved an initial denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 60 s, with fluorescence acquisition in single mode. After every 5 samples, *T. whipplei*–negative controls (water, mixture, and human samples) were evaluated.

If the result of the first PCR was positive, it was systematically confirmed by a second PCR with a second set of primer pairs: TW13 forward (5′-TGA GTGATGGTAGTCTGAGAGATATGT-3′) and TW163 reverse (5′-TCCATAACAAAGACAACAACCAATC-3′). This second PCR used a Taqman probe (13 forward–163 reverse, 5′-6-FAM-AGAAGAAGATGTTACGGGTTG-TAMRA-3′) specific for a different 150-bp sequence, as described elsewhere; the same amplification conditions described above were used. For quantitative PCR, sequence-specific standard curves were generated by using 10-fold serial dilutions of a standard concentration of 10^6 microorganisms of the Marseille-Twist *T. whipplei* strain. The number of transcript copies in each sample was then calculated from the standard curve by using LightCycler software.

Genotyping of *T. whipplei* from stool specimens was performed as reported (*19*). This analysis was specific for 4 highly variable genomic sequences (HVGS) and used primers TWT133 forward and reverse for HVGS 1, primers ProS forward and reverse for HVGS 2, primers SECA forward and reverse for HVGS 3, and primers TWT183 forward and reverse for HVGS 4. PCR was performed in a PTC-200 automated thermal cycler (MJ Research, Waltham, MA, USA), as reported (*19*).

Serologic assays were performed by using Western blotting as described (*29*). Before blotting, protein concentration was determined by using a commercial reagent (Bio-Rad, Hercules, CA, USA). *T. whipplei* Twist proteins were resuspended in Laemmli buffer (Sigma-Aldrich Chimie, Saint Quentin Fallavier, France) containing 100 mmol/L dithiothreitol to obtain a final protein concentration of 0.5 μg/μL. The protein lysate was heated for 5 min at 100°C. Five micrograms of native protein was loaded into wells of a 7.5% polyacrylamide gel, and proteins were resolved by sodium dodecyl sufate–polyacrylamide gel electrophoresis.

Proteins were then transferred to nitrocellulose membranes (Transblot Transfer Medium, Pure Nitrocellulose Membrane, 0.45 mm; Bio-Rad) over a 2-hour period by using a semidry transfer unit (Hoeffer TE 77; GE Healthcare, Little Chalfont, UK). Membranes were immersed in phosphate-buffered saline supplemented with 0.2% Tween 20 and 5% non-fat dry milk (blocking buffer) for 1 h at room temperature and incubated with primary serum (dilution 1:1,000 in blocking buffer) for 1 h at room temperature. Membranes were then washed in triplicate with phosphate-buffered saline–Tween 20, and immunoreactive spots were detected by incubating the membranes for 1 h at room temperature with peroxidase-conjugated goat anti-human antibody (Southern Biotech, Birmingham, AL, USA) diluted 1:1,000 in blocking buffer. The first screening was performed by testing for all immunoglobulins (Igs). Positive cases were then tested to separately detect IgG and IgM. Detection was performed by using chemiluminescence (Enhanced Chemiluminescence Western Blotting Analysis System; Amersham Biosciences, Uppsala, Sweden) with an automated film processor (Hyperprocessor; GE Healthcare).

Statistical Analysis

Data were analyzed by using EpiInfo software version 3.4.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Proportions were compared by using the Yates χ^2 corrected test or Fisher exact test. Continuous variables were compared by using analysis of variance or the Mann-Whitney/Wilcoxon 2-sample test when data were not normally distributed. Significance was defined as $p<0.05$.

Results

A total of 241 children 2–4 years of age with gastroenteritis were tested, and samples from 36 (15%) were positive for *T. whipplei*. In 2006, 2007, and 2008, the infection rates for *T. whipplei* were comparable: 12/78 (15.4%), 10/72 (14%), and 14/91 (15.4%), respectively. No seasonal variation was observed. None of the children in the same age control group without diarrhea had samples positive for *T. whipplei* (0/47; $p = 0.008$). High bacterial loads ($\geq 10^4$/g of stool) were observed in 64% (23/36) of the *T. whipplei*–positive children. Such high loads have not been observed in chronic carriers, but they were comparable to the levels for patients with Whipple disease (*14*). Bacterial load in stools ranged from 170 to 1.5×10^6/g (mean ± SD $1.5 \times 10^5 \pm 3.6 \times 10^5$) for children with gastroenteritis versus 85 to 2.5×10^6/g (mean ± SD $5.5 \times 10^5 \pm 8.3 \times 10^5$) for patients with Whipple disease ($p = 0.1$). Only 1 postdiarrheal stool specimen was slightly positive 15 days later and had a bacterial load <85/g of stool; the bacterial load was 2×10^4 at the time of diarrhea. Stool samples obtained from the same patient 1 month later were negative by PCR.

Genotyping of *T. whipplei* was performed for 34 children with diarrhea. A dendogram showing phylogenetic organization of genotypes is shown in Figure 1. We observed genetic heterogeneity in sequences associated with gastroenteritis and identified 12 new genotypes. One useful find-

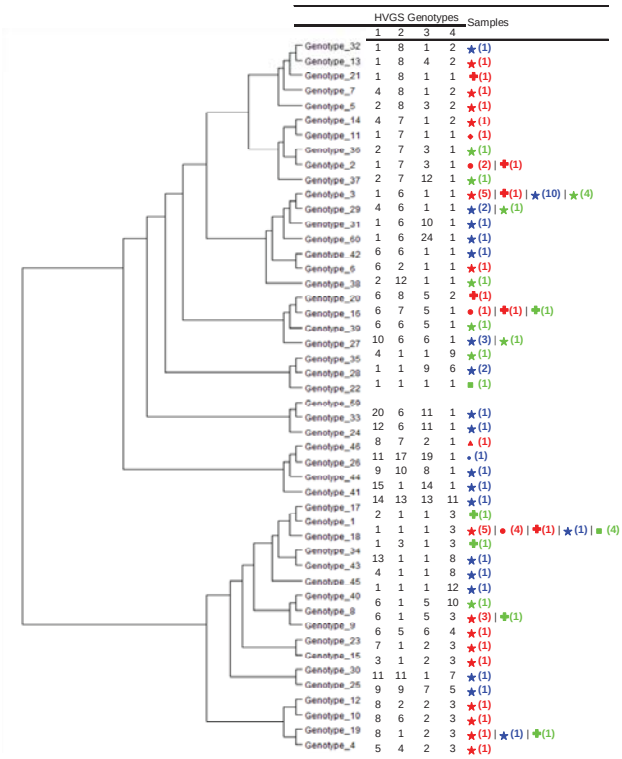

Figure 1. Dendogram constructed by using unweighted pair group method with arithmetic mean and 4 highly variable genomic sequences (HVGS), showing phylogenetic diversity of 48 genotypes of 81 *Tropheryma whipplei* strains detected in 34 children with diarrhea (blue), 40 adult patients with Whipple disease (red), and 22 asymptomatic adult patients without Whipple disease (green) (including 11 sewer workers), Marseille, France. Sequences were concatenated to construct the dendrogram. Numbers in parentheses indicate number of genotyped samples for each category. Stars, France; crosses, Switzerland; circles, Germany; diamond, Italy; square, Austria; triangle, Canada; small circle, Comoros.

ing was that genotype 3 was detected in 10 of the 34 children. Using Western blotting, we found that case-patients with Whipple disease were significantly more likely to be seropositive than controls with diarrhea (Figure 2) for IgG (7/8 vs. 5/25; p = 0.001) and IgM (7/8 vs. 1/25; p<0.001) and than controls without gastroenteritis for IgG (5/20; p = 0.004) and IgM (1/20; p<0.001).

Clinical and biological features of the 36 PCR-positive children 2–4 years of age with diarrhea were compared by retrospective review with those of matched 67 control children of the same age who had gastroenteritis but were negative for *T. whipplei* by PCR. Results are summarized in Table 1. Among children with positive PCR results, the proportion of girls and boys was equal. *T. whipplei* PCR-positive patients with diarrhea had a milder illness than PCR-negative patients. Length of rehydration required, duration of fever, and duration of hospitalization were significantly shorter for the patients infected with *T. whipplei* (p = 0.003, p = 0.003, and p = 0.01, respectively). Moreover, the level of C-reactive protein was significantly lower incase-patients (p = 0.03). Patients with *T. whipplei* were less likely to experience anorexia (p = 0.03); however, their weight loss was significant (p = 0.045). Another significant difference was increased contact with sand boxes for case-patients (p = 0.002); however, the size of the group analyzed was small, and these data should be confirmed.

Children infected with *T. whipplei* were co-infected with an associated pathogen more often than patients with diarrhea without *T. whipplei* infection (13/36 vs. 36/205; p = 0.01) (Table 2). Co-infection was less common in 23 children with high *T. whipplei* bacterial loads ($\geq 10^4$/g of stool) than in 13 children with lower bacterial loads (5/23 vs. 8/13; p = 0.02).

Discussion

T. whipplei has been identified by PCR in stools of persons without Whipple disease (*20*). The source of *T. whipplei* is unknown, but data suggest that it the infection may result from fecal–oral or oral–oral transmission (*2*,*20*). *T. whipplei* is excreted in a live form by patients with Whipple disease (*9*), and *T. whipplei* DNA is found in stool samples of healthy persons (*22*). This bacterium is commonly found

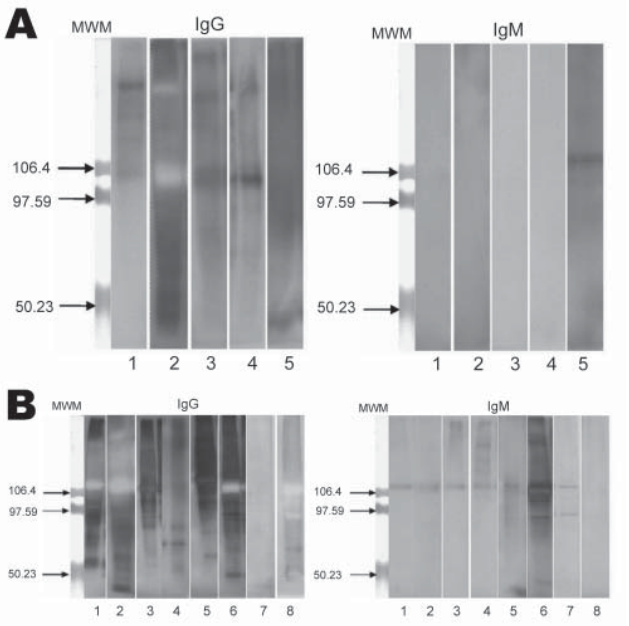

Figure 2. Western blot analysis of immunoglobulin (Ig) G and IgM against *Tropheryma whipplei* for children with gastroenteritis, Marseille, France. Total native antigens from *T. whipplei* were tested. A) Five patients without *T. whipplei* detected from stool samples but with positive Western blot serologic results. B) Eight patients infected with *T. whipplei*. MWM, molecular weight markers. Values on the right of each blot are in kilodaltons.

Table 1. Clinical and biological characteristics and laboratory test results of children 2–4 years of age with gastroenteritis who tested positive by PCR for *Tropheryma whipplei*, compared with controls, Marseille, France*

Characteristic	*T. whipplei* PCR positive, n = 36	*T. whipplei* PCR negative, n = 67†	p value
Male sex	19 (53)	35 (52)	0.9
Median age, mo	29 ± 6	29 ± 5	0.7
Sand box contact	4/4 (100)	0/8 (0)	0.002
Anorexia	14 (39)	41 (62)	0.03
Weight loss, kg	0.9 ± 0.6	0.55 ± 0.35	0.045
Vomiting	20 (55.5)	45 (67)	0.2
Abdominal pain	9 (25)	14 (21)	0.3
Dehydration	11 (30.5)	25 (37)	0.5
Watery diarrhea	25 (69)	46 (69)	0.9
Bloody diarrhea	3 (8)	5 (7.5)	0.9
Duration of diarrhea, d	4.5 ± 4	4.5 ± 4	0.9
No. stools/d	6 ± 3	5.5 ± 3	0.2
Length of rehydration required, h	19 ± 10	35 ± 23	0.003
Fever, °C	38 ± 0.9	38.3 ± 1	0.2
Duration of fever, d	1.4 ± 1	3.6 ± 3.7	0.003
Duration of hospitalization, d	1.6 ± 1.5	3.5 ± 3.8	0.01
Peripheral leukocyte count, × 10^9/L	10 ± 4	14 ± 7	0.2
Neutrophil count, × 10^9/L	6 ± 3	7.5 ± 6	0.7
Hemoglobin, g/dL	11.8 ± 1.5	11.9 ± 1.5	0.6
Fibrin, g/L	4 ± 0.8	4.2 ± 1.2	0.6
C-reactive protein, mg/mL	24 ± 23	64 ± 87	0.03
Serum albumin, g/L	37 ± 50.2	34 ± 3	0.2
Serum creatinine, mmol/L	37 ± 11	37 ± 12	0.99
Uremia, mmol/L	3.4 ± 2	3.4 ± 2	0.99
Proteinemia, g/L	69 ± 8	68.5 ± 7	0.7
Serum potassium, mmol/L	3.8 ± 0.5	3.9 ± 0.4	0.3
Partial thromboplastin time, s	31 ± 3	32 ± 5	0.6
Prothrombin time, s	83 ± 20	81 ± 17	0.7

*Values are no. (%), no. positive/no. tested (%), or mean ± SD.
†Among 205 case-patients with gastroenteritis who were negative for *T. whipplei*, data were available for 67 patients.

in sewers (*20*,*23*) and has also been detected in sewage (*25*). Therefore, it appears that *T. whipplei* is much more common than previously believed. A total of 3.8% (7/299) of adult controls in our study were PCR positive for *T. whipplei* (*14*). The bacterial load of *T. whipplei* in stools is much lower in asymptomatic carriers than in patients with Whipple disease (*14*,*20*).

We believe that our data provide strong evidence to support our initial hypothesis that *T. whipplei* causes mild gastroenteritis in children 2–4 years of age. All of our case-patients had 2 quantitative PCR-positive results, and genotyping of *T. whipplei* from 32 children enabled us to identify 12 new genotypes. Our data exclude the possibility of PCR contamination. We also found bacterial loads higher than those in previous reports of chronic carriers (*20*). However, these loads were comparable with those for patients with active Whipple disease (*14*). These high loads suggest that gastroenteritis in the children in our study was associated with active *T. whipplei* replication.

The absence of *T. whipplei* DNA in stools after patient recovery from diarrheal illness strongly suggests that detection of *T. whipplei* DNA is associated with acute gastroenteritis rather than carriage, which is usually chronic (*20*). Moreover, 1 genotype (genotype 3) predominated, causing one third of the total number of cases.

Serologic analysis by Western blotting for *T. whipplei* (*29*) showed that all children who were PCR positive for *T. whipplei* had IgM against *T. whipplei*, which suggested that recent seroconversion had occurred. The prevalence of antibodies in case-patients was higher than that in controls. Comparison of the prevalence of antibodies for control children (22%) with preliminary prevalence data for persons 61 ± 3.6 years of age (45%) (*30*) showed that the difference is significant ($p<0.001$); thus, possible acquired immunity to infection with *T. whipplei* may explain why this bacterium causes diarrhea in children, but not in most adults.

We suspect that *T. whipplei* infection is contagious and transmitted by the fecal–oral route in children 2–4 years of age with other enteric pathogens. We previously reported an association between *G. duodenalis* infection and Whipple disease (*27*). As with other organisms such as *Helicobacter pylori*, *T. whipplei* may also be transmitted through saliva because it has been found in saliva of asymptomatic carriers and patients with Whipple disease (*20*). Therefore, we hypothesized that primary infection with *T. whipplei* in

Table 2. Microbiologic data for stool specimens of 241 children, Marseille, France*

Pathogen	*T. whipplei*–positive children			*T. whipplei*–negative children
	Bacterial load $<10^4$/g of stool	Bacterial load $\geq 10^4$/g of stool	All	
Any other	8/13	5/23	13/36	36/205
Bacteria				
Salmonella sp.	1 (1 with *Giardia duodenalis*)	2	3 (1 with *G. duodenalis*)	2
Other	1 with *Campylobacter jejuni* (also associated with rotavirus)	0	1 with *C. jejuni* (also associated with rotavirus)	5 (1 *C. jejuni,* 1 *Escherichia coli* O26:B6, 1 *E. coli* O126:B16 with 1 rotavirus, 1 *Shigella sonnei,* and 1 *Yersinia enterocolitica*)
Viruses				
Rotavirus	5	3	8 (1 also associated with *C. jejuni*)	21
Other	2 (1 adenovirus and 1 calicivirus)	0	2 (1 adenovirus and 1 calicivirus)	9 (4 enterovirus, 3 adenovirus, and 2 calicivirus)

*Testing for *Tropheryma* whipplei was conducted by using PCR.

children may result in gastroenteritis, especially when associated with other intestinal pathogens. We have provided several lines of evidence that *T. whipplei* is causing or exacerbating gastroenteritis. The incidence of *T. whipplei* DNA in stools of children 2–4 years of age with gastroenteritis was higher than that in the control group; these infected children also have higher levels of antibodies. That healthy children of the same ages were not infected with *T. whipplei* also suggests that primary infection is symptomatic. Sociodemographic differences between the 2 groups could theoretically explain our findings. However, most of our patients and controls were from the same geographic area, which enabled us to rule out this hypothesis.

We identified 1 clone (genotype 3) in 10 children, which indicates that this clone is circulating in our population. The association we found between *T. whipplei* and other pathogens transmitted by the fecal–oral route supports the conclusion that *T. whipplei* and other intestinal pathogens have a common source of infection and that they are often co-transmitted (*31*,*32*). However, for unknown reasons, *T. whipplei* could replicate in children with low-grade chronic infections without causing diarrhea. That *T. whipplei*–infected patients were more likely to have co-infections than patients infected with other pathogens may also indicate that *T. whipplei* may decrease below molecular detection limits when diarrhea resolves. However, higher levels of IgM against *T. whipplei* in case-patients suggest infection with this bacterium. Moreover, an in vivo animal model of oral infection by *T. whipplei* has shown a pathogenic effect only in mice with previously inflamed colonic tissues (D. Raoult et al., unpub. data). Thus, inflamed colonic tissues may also explain the frequency of co-infections with common pathogens observed in persons with *T. whipplei*–positive gastroenteritis.

We provide evidence that *T. whipplei* is commonly associated with gastroenteritis in children. We also suggest that other studies should be performed to evaluate the role of this bacterium and its prevalence in patients with gastroenteritis because it is present worldwide.

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Dr Raoult is a physician and research scientist at the Unité des Rickettsies, Université de la Méditerranée, Marseille, France. His research interests include *Tropheryma whipplei* and Whipple disease.

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Contagious Period for Pandemic (H1N1) 2009

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We estimated the proportion of persons with pandemic (H1N1) 2009 who were shedding infectious virus at diagnosis and on day 8 of illness. In households with confirmed cases, nasopharyngeal swabs were collected on all members and tested by PCR and virus culture. Of 47 cases confirmed by PCR at <7 days of illness, virus culture was positive in 92% (11/12) of febrile and 63% (22/35) of afebrile persons. Of 43 persons with PCR-confirmed pandemic (H1N1) 2009 from whom a second specimen was collected on day 8, 74% remained PCR positive and 19% were culture positive. If the 73 symptomatic household members without PCR-confirmed illness are assumed to have pandemic (H1N1) 2009, a minimum of 8% (6/73) of case-patients shed replicating virus on day 8. Self-isolation only until fever abates appears insufficient to limit transmission. Self-isolation for a week may be more effective, although some case-patients still would shed infectious virus.

Since April 2009, an influenza A virus, pandemic (H1N1) 2009, has spread to most countries of the world. Widespread susceptibility of persons <60 years of age may have facilitated rapid dissemination (*1*). Transmissibility of influenza viruses depends on duration of shedding, amount of virus shed, and other factors that may facilitate projection of virus into the environment, such as coughing or sneezing. Challenge studies in healthy volunteers inoculated with seasonal influenza viruses have shown that shedding generally coincides with symptom onset starting 1 day after inoculation, peaks on the second day, and generally ends 1 week after disease onset, on day 8 (*2*). Duration of shedding is greatly affected by age, and for seasonal influenza viruses is longer in young children than in adults (*3*–*5*). Since the emergence of pandemic (H1N1) 2009, the recommended duration of self-isolation has varied from complete resolution of symptoms to 1 day after fever has subsided (*6*,*7*). The objective of this study was to estimate the proportion of pandemic (H1N1) 2009–infected persons shedding infectious virus 1 week after illness onset.

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Methods

Study Setting

The research ethics committee of the Centre Hospitalier Universitaire de Québec approved the study. The prospective study was conducted during May 27–July 10, 2009, in Quebec City, Quebec, Canada. All participants were community based. Eligible persons were members of households in which at least 1 person with pandemic (H1N1) 2009 confirmed by reverse transcription–PCR (RT-PCR). Primary case-patients were either referred by their treating physicians or identified among community contacts of persons with laboratory-confirmed cases (Figure 1). At the initial home visit, a nurse obtained written informed consent from all household members and collected data for each household participant by using a standardized questionnaire. The questionnaire asked about basic sociodemographic characteristics (e.g., age, sex, occupation), presence of underlying medical conditions, presence of various symptoms or signs (i.e., fever, chills, cough, sore throat, rhinorrhea, arthralgia/myalgia, fatigue,

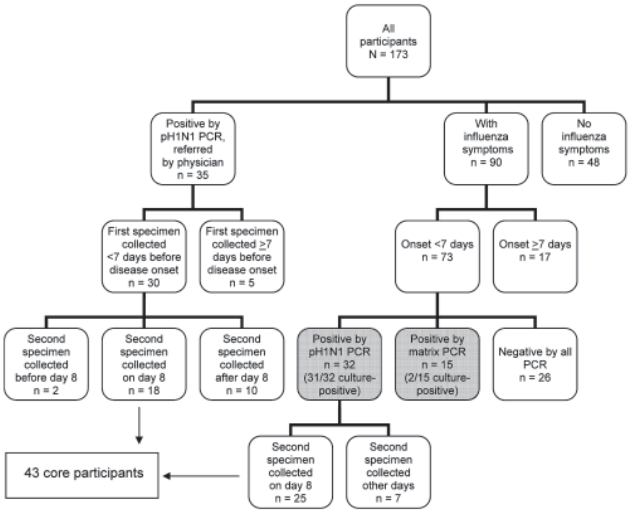

Figure 1. Flowchart of characteristics of 173 participants in study of shedding of pandemic (H1N1) 2009 virus, Quebec City, Quebec, Canada, May 27–July 10, 2009.

dyspnea, headache, diarrhea, or vomiting), seasonal influenza vaccination history, and social and healthcare impact of their illness (i.e., missed workdays or schooldays, days spent in bed, medical consultations, emergency department visits, or hospital admissions).

Nasopharyngeal Swabs and Laboratory Procedures

Nasopharyngeal (NP) secretions were collected from all household members, both symptomatic and asymptomatic, with NP swabs (Nylon Flocked Swabs; Copan Innovation, Brescia, Italy) that were inserted into 3 mL of universal transport medium (Copan Innovation). Patients with results positive by RT-PCR for pandemic (H1N1) 2009 before day 7 since disease onset had a second NP swab repeated on day 8. Persons still positive on day 8 were retested on day 11.

Nucleic acids were extracted from 200 μL of specimen by using the QIAGEN Viral RNA Mini Kit (QIAGEN, Mississauga, Ontario, Canada). Initially, pandemic (H1N1) 2009 was detected by using a conventional RT-PCR (pH1N1 PCR) specific for the hemagglutinin gene (*8*). RNA extracts from pH1N1 PCR–negative specimens were frozen at –80°C and, once specimen collection was completed, were retested by using a conventional RT-PCR assay for all influenza A viruses (matrix PCR), which has a higher sensitivity (*9*).

All samples with RT-PCR–positive results were cultured on MDCK cells in shell vials containing 1 mL of media to detect replicating viruses. Vials were observed daily for 7 days to detect cytopathic effects. Virus cultures were conducted on fresh specimens if they were positive by the pH1N1 PCR, whereas samples positive by the conventional matrix PCR had undergone 1 freeze-thaw cycle. All cultures showing cytopathic effects were sent to the Québec provincial reference laboratory for RT-PCR confirmation by using the previously described pH1N1 PCR.

Statistical Analyses

We compared proportions and distributions using the χ^2 test or Fisher exact test when appropriate. All statistical analyses were 2-tailed, and p values ≤ 0.05 were considered significant.

Results

Of the 173 persons from 47 participating households, 35 with pH1N1 PCR–confirmed persons (index case-patients) were referred by their treating physicians. Among the 138 other participants (household or community contacts), 73 had respiratory symptoms for <7 days at the time of enrolment and 32 (44%) of 73 were positive by pH1N1 PCR (Figure 1, Table 1). Cell culture was also positive for 97% (31/32) of these pH1N1 PCR–confirmed cases. Of the 32 pH1N1 PCR–confirmed cases, 78% (25/32) had fever at some point since illness onset; at the time of specimen collection, 94% (30/32) had cough and 34% (11/32) were still febrile. Specimens were retested with a matrix PCR. All results from the pH1N1 PCR–positive participants were also positive by matrix PCR; of those who were negative by pH1N1 PCR, 37% (15/41) were positive. Of the 15 participants positive by matrix PCR, 13% (2/15) were positive by cell culture. Of the 47 confirmed cases (32 by pH1N1 PCR and 12 by matrix PCR), cell culture positivity varied from 69% to 87% for specimens obtained within 4 days after symptom onset and dropped to 33%–40% for specimens obtained days 5 and 6 after symptom onset (Figure 2).

Of the 67 patients whose results were positive by pH1N1 PCR (35 case-patients initially referred by their physicians plus 32 detected among contacts), 62 were identified ≤7 days after symptom onset and 43 had a second swab collected on day 8 (Figure 1). Of these 43 cases (core participants), 47% were children <10 years of age, 26% were 10–17 years of age, and 28% were adults (≥18 years of age) (Table 1). On day 8, 18 (42%) were still positive by pH1N1 PCR, and 14 others were positive only by matrix PCR, for a total of 74% (32/43) positive by any PCR method on day 8. (Table 2) Virus culture was positive for 19% (8/43) of the patients: culture was positive only for pH1N1 PCR–positive cases (8/18, 44%) but for none of the cases positive only by matrix PCR. PCR and virus culture positivity rates did not differ among age groups (Table 2). Only 5% (2/43) of case-patients were still febrile on day 8, but 91% (39/43) were still coughing. None of the 8 patients who had a positive virus culture on day 8 were febrile, but 7 (88%) were still coughing. Another swab was repeated on day 11 for 16 of the 18 case-patients who were positive by

Table 1. Characteristics of various subgroups of participants in assessment of length of shedding of pandemic (H1N1) 2009 virus, Quebec City, Quebec, Canada, May 27–July 10, 2009*

Characteristic	No. (%) core participants, n = 43	Symptomatic household or community contacts tested <7 days after symptom onset			
		Total no. (%), n = 73	No. (%) positive by pH1N1 and matrix PCRs, n = 32	No. (%) positive by matrix PCR only, n = 15	No. (%) negative by both PCRs, n = 26
Age group, y					
<10	20 (47)	24 (33)	13 (41)	4 (27)	7 (27)
10–17	11 (26)	15 (21)	9 (28)	1 (7)	5 (19)
≥18	12 (28)	34 (47)	10 (31)	10 (67)	14 (54)
Sex					
F	24 (56)	41 (56)	17 (53)	8 (53)	16 (62)
M	19 (44)	32 (44)	15 (47)	7 (47)	10 (38)
Medical condition					
Any influenza-associated	8 (19)	15 (21)	6 (19)	4 (27)	5 (19)
Pulmonary disease	4 (9)	10 (14)	3 (9)	4 (27)	3 (12)
Influenza vaccination					
2008–2009 season	9 (21)	15 (21)	8 (25)	6 (40)	1 (4)
Clinical Illness†					
Fever	37 (86)	37 (51)	25 (78)	8 (53)	4 (15)
Cough	42 (98)	58 (79)	31 (97)	13 (87)	14 (54)
Sore throat	26 (60)	41 (56)	19 (59)	5 (33)	17 (65)
Myalgia or arthralgia	17 (40)	23 (32)	12 (38)	4 (27)	7 (27)
Fatigue	40 (93)	51 (70)	31 (97)	10 (67)	10 (38)
Headache	31 (72	45 (62)	24 (75)	9 (60)	12 (46)
Gastrointestinal symptoms	14 (33)	18 (25)	8 (25)	5 (33)	5 (19)
Fever and cough	36 (84)	35 (48)	24 (75)	8 (53)	3 (12)
Influenza-like illness	36 (84)	34 (47)	24 (75)	8 (53)	2 (8)

*pH1N1 PCR, PCR for pandemic (H1N1) 2009 virus.
†Symptoms between onset of disease and first nasopharyngeal swab.

pH1N1 PCR on day 8 and 14 (88%) were still positive by at least 1 PCR (4 by pH1N1 PCR and 10 by matrix PCR). However, no specimen was positive by cell culture. On day 11, a total of 12 (86%) of the 14 PCR–positive case-patients were still coughing.

Of the 73 symptomatic participants tested within 7 days after symptom onset, 15 of the 47 PCR–positive case-patients (in gray in Figure 1) were detected only by matrix PCR (2/15 cell culture positive) (Table 1). Of specimens positive by pH1N1 PCR, 97% (31/32) were culture positive at diagnosis, 44% (8/18) on day 8, and none on day 11 of illness. Of those whose results were positive by matrix PCR, 13% (2/15) were culture positive at diagnosis, and none were positive on days 8 or 11 of illness. Because virus culture was much less frequently positive for specimens positive only by matrix PCR (2/39, 5%) than for specimens positive by pH1N1 PCR (39/54, 72%), the 19% virus culture positivity on day 8 among core participants (all pH1N1 PCR positive) overestimated the true proportion positive at day 8. Assuming that none of the 15 case-patients with matrix PCR–positive results and 19% of the 32 case-patients with pH1N1 PCR–positive results would shed live virus on day 8, we can estimate that 6 [(19% × 32 pH1N1 PCR positive) + (0 × 15 matrix PCR positive)] of the 47 (13%) case-patients would still be positive by virus culture on day 8. If, instead, we assume that all 73 symptomatic participants were infected by pandemic (H1N1) 2009 and that positive cell culture on day 8 would be found only in patients positive by pH1N1 PCR, then 8% (6/73) still would be shedding live virus 1 week after illness onset. The real cell culture positivity rate on day 8 for all pandemic (H1N1) 2009–infected patients thus probably ranges from 8% to 13%.

Discussion

Human challenge studies with seasonal influenza have shown that virus shedding after day 7 is rare (*2*), but clinical studies have shown that shedding may persist beyond that period in some populations, such as elderly persons, immunocompromised patients, and children (*3–5*,*10–12*). In a study among hospitalized persons infected with seasonal influenza A viruses, 54% remained positive by PCR beyond 7 days after symptom onset, and 29% were positive by cell culture (*13*). In another study, elderly hospitalized patients infected by influenza A (H3N2) viruses had higher virus loads than did outpatients, and their PCR positivity rate 1 week after disease onset was still high (57%) (*10*).

In this prospective study, the proportion of pandemic (H1N1) 2009–infected persons still shedding replicating virus on day 8 varied from 8% to 13%, with no difference between children and adults. None were still shedding infectious virus on day 11. With seasonal influenza, virus

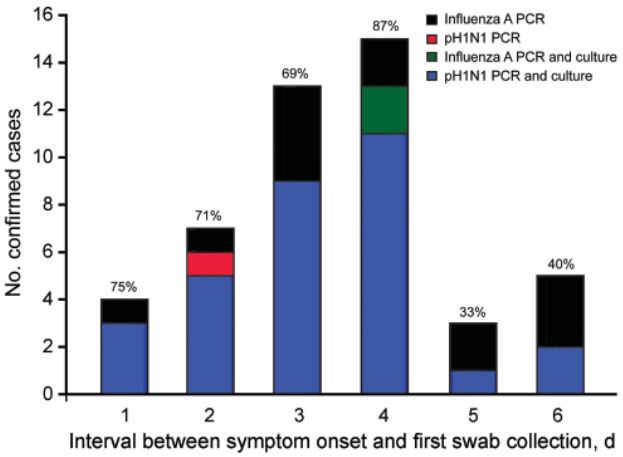

Figure 2. Positive results by PCR and culture for influenza A and pandemic (H1N1) 2009 virus (pH1N1) in 47 household contacts with laboratory-confirmed influenza, by delay between day of symptom onset and day of first swab collection, Quebec City, Quebec, Canada, May 27–July 10, 2009.

shedding may be longer in children because they have less preexisting immunity that would limit replication than in adults. However, children and adults <50 years of age appear equally susceptible to infection with pandemic (H1N1) 2009 virus, which would support our findings of comparable virus replication and shedding across age groups studied (*1*).

Our study had some limitations. First, our small sample size and study design may have limited our ability to directly measure culture positivity on day 8. Only a small number of patients had a specimen collected on day 8 and even fewer on day 11. In retrospect, a better design would have been to collect specimens from all 73 symptomatic household members on day 8, irrespective of the initial pH1N1 PCR result. That design would have enabled a more direct estimate of the proportion of patients who were culture positive on day 8, rather than the indirect approach we used. However, our extreme scenario (which assumes that all 73 symptomatic contacts were infected) provides the minimal positivity rate on day 8, and testing of all 73 on day 8 could only have found a proportion equal to or greater than our 8% estimate.

Second, our sampling methods could have influenced positivity rates. Although collection of NP specimens with a flocked swab is one of the best methods for obtaining specimens to detect influenza, those specimens might have been improperly collected by the nurses. Suboptimal collection of swabs would have yielded false-negative PCR or cell culture results, which in turn would have underestimated the proportion of patients shedding virus on day 8.

Third, PCR testing with the matrix PCR was conducted retrospectively on frozen specimens, and only 5% of those were positive by virus culture. A greater proportion of virus culture specimens might have been positive if those specimens had been processed immediately instead of going through a freeze-thaw cycle (*14*). Moreover, our study included only ambulatory patients ,whereas studies of seasonal influenza that include hospitalized or immunocompromised persons show prolonged shedding, contributing to the impression that our findings most likely underestimate the true proportion of case-patients still shedding virus on day 8. The strengths of our study include its prospective design in a family setting and its use of various methods, including 2 PCR assays and virus culture, to detect pandemic (H1N1) 2009.

Our results are consistent with other reports of virus shedding in pandemic (H1N1) 2009–infected patients. In Singapore, among 70 pandemic (H1N1) 2009–infected patients treated with oseltamivir and swabbed daily until virus

Table 2. PCR and virus culture positivity on days 8 and 11 of illness, by age group, Quebec City, Quebec, Canada, May 27–July 10, 2009*

	Age group, no. (%)				
Time after illness onset	0–9 y	10–17 y	≥18 y	Total no. (%)	p value
Day 8 of illness (2nd swab)	n = 20	n = 11	n = 12	n = 43	
pH1N1 PCR–positive specimen	10/20 (50)	3/11 (27)	5/12 (42)	18/43 (42)	0.43
Culture positive on pH1N1 PCR–positive specimen	5/10 (50)	1/3 (33)	2/5 (40)	8/18 (44)	1.00
Matrix PCR positive	5/10 (50)	6/8 (75)	3/7 (43)	14/25 (56)	0.53
Culture positive on matrix PCR–positive specimen	0/5	0/6	0/3	0/14	–
Any PCR positive	15/20 (75)	9/11 (82)	8/12 (67)	32/43 (74)	0.82
Culture positive on any PCR–positive specimen	5/15 (33)	1/9 (11)	2/8 (25)	8/32 (25)	0.51
Day 11 of illness (3rd swab)	n = 8	n = 3	n = 5	n = 16	
pH1N1 PCR–positive specimen	3/8 (38)	0/3	1/5 (20)	4/16 (25)	0.77
Culture positive on pH1N1 PCR–positive specimen	0/3	0/0	0/1	0/4	–
Matrix PCR–positive specimen	4/5 (80)	3/3 (100)	3/4 (75)	10/12 (83)	1.00
Culture positive on matrix PCR positive–specimen	0/4	0/3	0/3	0/10	–
Any PCR positive–specimen	7/8 (88)	3/3 (100)	4/5 (80)	14/16 (88)	1.00
Culture positive on any PCR positive–specimen	0/7	0/3	0/4	0/14	–

*pH1N1 PCR, PCR for pandemic (H1N1) 2009 virus.

clearance, 37% were PCR positive on day 7 of their illness and 9% on day 10 (*15*). No virus culture was performed in that study, so we cannot estimate the proportion of patients shedding infectious virus at these time points. However, even with oseltamivir treatment, the positivity rate by pH1N1 PCR on day 7 was similar to our own (42%) on day 8, and we can thus infer that the cell culture positivity rates also would be similar. In China, among 421 patients with serial swabs tested by real-time PCR but not cell culture, the median time from onset of disease to negative test result by real-time PCR was 6 days (range 1–17 days), indicating that 50% of patients were shedding virus ≥6 days (*16*).

A study conducted by Witkop et al. during a pandemic (H1N1) 2009 outbreak at the US Air Force Academy showed that 29% (31/106) of afebrile patients and 19% (11/58) of patients who had been symptom-free for 24 hours still shed viable pandemic (H1N1) 2009 virus. In their study, 24% of 29 swabs collected on day 7 and 13% of the 16 swabs collected on day 8 of illness were culture positive, despite the large proportion of patients prescribed antiviral drugs (*17*).

No definitive test is available for assessing the real contagiousness of a patient. The presence of replicating, and therefore infectious, influenza virus is an absolute prerequisite for contagiousness, but it does not necessarily imply it. Contagiousness depends on many factors, including viral load and presence of clinical characteristics contributing to spread of droplets (such as coughing, rhinorrea, or sneezing) and is affected by the number and proximity of contacts between a case-patient and a susceptible person. Nevertheless, our study raises concerns about current recommendations for self-isolation until only 24 hours after fever has subsided (*6*). With pandemic (H1N1) 2009, fever generally persists 1–4 days and may be absent in 6%–11% of patients (*1*,*15*). In our study, of the 32 pH1N1 PCR–positive household members who had been symptomatic for <7 days, 78% had fever at any time since onset of their illness, but only 34% were still febrile on the day they tested positive. Nonetheless, 97% of specimens obtained from these patients were positive by cell culture. Our sample size was insufficient to directly compare PCR or culture positivity by fever status or other symptom or severity indicator at specimen collection or as a component of the overall illness.

Before policy implications can directly follow from these findings, the association of self-isolation with substantial social impact needs to be carefully weighed against the possible benefits of reducing community transmission. In the general population, a 1-week self-isolation period seems more likely to prevent transmission than does isolation until fever has resolved. However, given that 8%–13% of patients may still shed infectious virus on day 8, longer periods of self-isolation for persons expected to come into contact with vulnerable persons (e.g., pregnant women, newborns, or immunocompromised persons) also may be prudent.

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Historical Distribution and Molecular Diversity of *Bacillus anthracis*, Kazakhstan

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To map the distribution of anthrax outbreaks and strain subtypes in Kazakhstan during 1937–2005, we combined geographic information system technology and genetic analysis by using archived cultures and data. Biochemical and genetic tests confirmed the identity of 93 archived cultures in the Kazakhstan National Culture Collection as *Bacillus anthracis*. Multilocus variable number tandem repeat analysis genotyping identified 12 genotypes. Cluster analysis comparing these genotypes with previously published genotypes indicated that most (n = 78) isolates belonged to the previously described A1.a genetic cluster, 6 isolates belonged to the A3.b cluster, and 2 belonged to the A4 cluster. Two genotypes in the collection appeared to represent novel genetic sublineages; 1 of these isolates was from Krygystan. Our data provide a description of the historical, geographic, and genetic diversity of *B. anthracis* in this Central Asian region.

Anthrax is a globally widespread disease of livestock and wildlife that occasionally infects humans. According to official estimates, the number of human anthrax cases worldwide ranges from 2,000 to 20,000 annually (*1*). *Bacillus anthracis*, the etiologic agent of anthrax, persists in the environment as a dormant, highly stable spore. The prolonged periods of dormancy during the spore phase slows evolution of this species, resulting in high levels of interstrain genetic homogeneity, which complicates efforts to subtype the pathogen. The availability of whole-genome nucleotide sequences of *B. anthracis* for single-nucleotide polymorphism (SNP) elucidation and the discovery of polymorphic markers such as variable number tandem repeat (VNTR) sequences (*2*,*3*) have enabled identification of unique subtypes within this species. Keim et al. (*4*) used 8 VNTRs to describe 89 unique genotypes in a global collection of over 400 *B. anthracis* isolates. Later studies used VNTRs to examine *B. anthracis* diversity in different global regions, including France (*5*), Italy (*6*), Poland (*7*), Chad (*8*), and South Africa (*9*). More recently, SNPs that define major clonal lineages in *B. anthracis* have been identified and applied to describe global and regional patterns of *B. anthracis* diversity (*10*).

In the central Asian republic of Kazakhstan, anthrax is enzootic and still represents a human public health concern. A recent publication examined risk factors associated with 73 human anthrax cases in Kazakhstan over a 2-year period (*11*) and concluded that most cases were cutaneous and had resulted from the handling of infected livestock and contaminated animal products. Gastrointestinal anthrax in Kazakhstan has also been reported but is less common. Despite the widespread nature of the disease in this region, the historical incidence, distribution, and genetic diversity of

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B. anthracis in central Asia, and Kazakhstan in particular, has remained cryptic.

We mapped the historical distribution of anthrax in Kazakhstan over a 68-year period. Archived cultures from a subset of these outbreaks collected from 10 oblasts (provinces) over a 53-year period were analyzed by using genetic and biochemical tests. Multilocus variable number tandem repeat analysis (MLVA) and canonical single nucleotide polymorphism genotyping (*10*) of this collection enabled us to examine strain dynamics among and within these outbreaks and to understand the diversity of *B. anthracis* isolates from Kazakhstan on a local, regional, and global scale.

Materials and Methods

Mapping Historical Anthrax Outbreaks

To map the historical distribution of anthrax outbreaks and *B. anthracis* strain types across Kazakhstan, we constructed a geographic information system (GIS) database within ArcGIS 9.1 (www.esri.com). This database used archival data collected through the antiplague stations established by the Union of Soviet Socialist Republics. This system of stations remains in place under the current government, and Kazakhstan maintains a multiagency reporting protocol to update, document, and respond to the distribution of outbreaks. These data are archived at the Kazakhstan Scientific Center for Quarantine and Zoonotic Diseases. Outbreaks and strain locations were geolocated to the nearest village by using GIS data layers produced by the Kazakh Institute of Geography. Historical outbreaks were mapped for 1937 through 2005. To illustrate differences in the distributions of outbreaks in cattle and sheep, the 2 most affected livestock species, a kernel density estimation was performed by using the Spatial Analyst Extension in ArcGIS. We mapped outputs by using the standard deviation of density values to illustrate areas of greatest outbreak concentration by species (*12*).

Isolation of *B. anthracis*

Samples collected from anthrax outbreaks in Kazakhstan (with the exception of 2 isolates from the Kyrgyzstan border region) and cultures spanning a 53-year period were archived in the Kazakhstan National *B. anthracis* Collection. Most isolates were from human patients, some from blood or organs of ruminants (mainly sheep and cows), and a few from soil or other inanimate objects contaminated by contact with blood or tissues of infected animals. Archived cultures were confirmed as *B. anthracis* on the basis of colony morphologic appearance; absence of hemolysis and catalase, lipase, phosphatase and protease activity; and susceptibility to *B. anthracis*–specific γ phage.

DNA Preparation

B. anthracis cultures from the Kazakhstan National Collection were grown on Hottinger blood agar. A colony from each sample was harvested from the agar plates and dispersed in Tris-EDTA buffer for DNA extraction. A QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) was used to extract genomic and plasmid DNA by using the manufacturer's protocol. A total of 1.0 mL of DNA was collected from each of the isolates in the collection.

MLVA Genotyping

Eight VNTR (MLVA-8) markers were amplified by PCR by using primer pairs *vrrA*-f1 and *vrrA*-r1, $vrrB_1$-f1 and $vrrB_1$-r1, $vrrB_2$-f1 and $vrrB_2$-r1, $vrrC_1$-f1 and $vrrC_1$-r1, $vrrC_2$-f1 and $vrrC_2$-r1, CG3-f1 and CG3-r1, pXO1-AAT-f3 and pXO1-AAT-r3, and pXO2-AT-f1 and pXO2-AT-r1 (*4*). One microliter containing ≈1 ng of template DNA was added to each PCR.

Electrophoresis of amplified products was performed on an ABI 310 genetic analyzer (Applied Biosystems, Inc., Foster City, CA, USA). Data were analyzed by using GeneMapper software V4.0 (Applied Biosystems, Inc.). To ensure comparability and accuracy of raw VNTR scores from the strains from Kazakhstan with the genotypes published by Keim et al. 2000 (*4*), we performed electrophoresis on amplified fragments from 4 control DNAs (A0462-Ames, A0488-Vollum; A0071-Western North America and A0402; and French B2) in parallel with the isolates from Kazakhstan. In addition, DNA molecular size reference markers (Applied Biosystems, Inc) were included in each sample to accurately size the 8 VNTR fragments. Raw VNTR sizes were normalized to the sizes reported by Keim et al., 2000 (*4*) for genotypic comparisons.

Unweighted Pair Group Method with Arithmetic Mean Cluster Analysis of Genotypes

Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of VNTR data from 92 confirmed *B. anthracis* isolates and the diverse 89 genotypes described by Keim et al. 2000 (*4*) were used to establish genetic relationships. Distance matrices were generated in PAUP 4.0 (Sinauer Associates, Inc., Sunderland, MA, USA) and imported into MEGA 3.1 (*13*) for tree-building purposes.

Spatial Patterns of Genetic Relationships

The strain database was constructed from museum records and contemporary epidemiologic investigations. This database was synchronized with the bacterial culture collection to geolocate the culture by using the GIS. To map strain diversity, we categorized culture collection locations by strain identifications based on the MLVA genotyping results.

SNP Typing of *B. anthracis* Isolates

Representative cultures from each Kazakh MLVA genotype plus the STI vaccine strain from Russia were genotyped by using previously described canonical SNPs discovered by whole-genome sequencing (*10*,*14*). SNPs were interrogated by using the Roche Light Cycler II real-time PCR instrument (Roche Diagnostics, Indianapolis, IN, USA). Allelic discrimination assays initially developed on the ABI 7900 real-time platform (*10*) were adapted for use on the Light Cycler II. The assay amplifies a fragment of DNA sequence containing the SNP site. Two probes complementing the 2 potential SNP states were used as real time markers. Each probe had a distinct fluorescent label; i.e., probe 1 was labeled with 6-carboxyfluorescein, and the alternate probe was labeled with VIC (Applied Biosystems, Inc.). The probe complementary to the sequence in the sample amplicon will hybridize over the SNP and surrounding sequence during the amplification process to generate a signal. It is possible for the incorrect probe to generate some signal but not enough to be confused as a positive reaction. The Light Cycler II discriminated which probe was the complementary sequence on the basis of the differential intensity of the reaction. Controls for each run included template DNA with both SNP states of interest.

Results

Historical Incidence and Geographic Distribution of Anthrax in Kazakhstan

A total of 1,037 human outbreaks were reported, representing 1,765 human cases. The outbreaks occurred in 665 locations; 198 of those locations reported repeat outbreaks throughout the study period (Figure 1; Table 1). Additional review of historical data at the Kazakhstan Scientific Center for Quarantine and Zoonotic Diseases identified 3,947 outbreak events reported for animal species and were entered into GIS. The outbreaks occurred over 1,790 locations; 805 of those reported repeated outbreaks.

Table 1. Outcomes for 1,765 human patients in mapped anthrax outbreak areas, Kazakhstan, 1937–2005

Status	Number
Recovered	1,541
Deceased	75
Lost contact	17
No data/unknown	132

Cattle and sheep were the primary livestock species affected during the study period; fewer outbreaks occurred among swine, and rarer, sporadic outbreaks occurred on mink farms and among foxes, and camels (Table 2). Cattle outbreaks were most common in northern Kazakhstan; several outbreaks occurred in the southernmost oblasts bordering Uzbekistan and Kyrgyzstan (Figure 2, panel A). Sheep outbreaks were prominent throughout eastern and southern Kazakhstan (Figure 2, panel B). The largest cattle outbreak (n = 174 cattle) in the dataset occurred in 1957 in the northernmost region of the Karaganda oblast in north central Kazakhstan. The largest sheep outbreak affected 851 sheep and occurred in the southern oblast of Zhambyl in 1971.

Biochemical Tests

All cultures except 1 (isolate no. 49) were biochemically and morphologically consistent for *B. anthracis*; 3 cultures (isolate nos. 65, 76, and 77) were consistent with *B. anthracis* but did not exhibit capsule formation. With the exception of culture no. 49, isolates were nonhemolytic; nonmotile; phosphatase and lecithinase negative; protease, oxidase, and catalase positive; and, with 3 exceptions, formed a capsule.

MLVA Genotyping

Of the 92 *B. anthracis* isolates, 88 isolates yielded complete data for the 8 marker MLVA; 3 isolates were missing the pX02 marker (isolate nos. 65, 76, and 77), and 1 was missing the pX01 plasmid marker (isolate no. 7). After we coded the raw VNTR fragment sizes, the Kazakh *B. anthracis* genotypes were analyzed by using PAUP 4.0

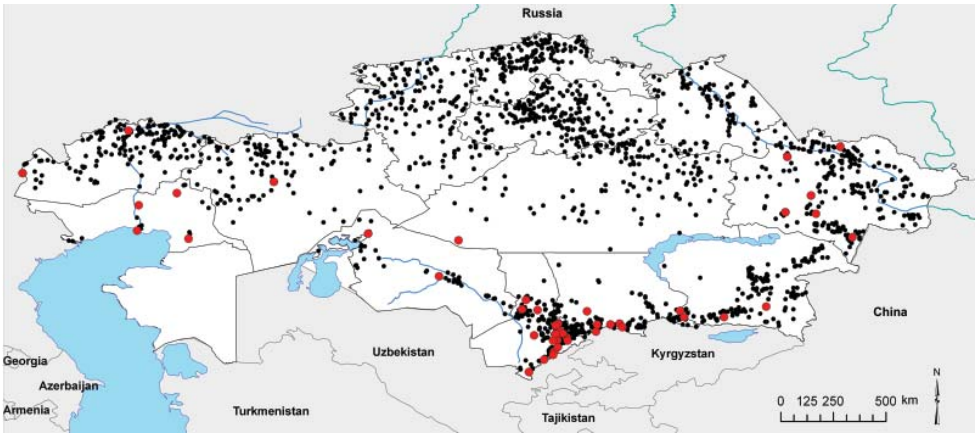

Figure 1. Anthrax outbreaks in Kazakhstan, 1937–2005. Each dot represents an outbreak; red dots indicate that cultures were isolated and analyzed from these outbreaks.

Table 2. Anthrax outbreaks, number of animal deaths per outbreak by species affected, and miscellaneous anthrax-positive samples, Kazakhstan, 1937–2005

Animal species	No. outbreaks/ samples	Deaths/ outbreak*	Total no. deaths
Sheep	1,735	0–851	16,080
Cattle	1,678	0–84	3825
Equine	304	0–28	634
Swine	192	0–78	832
Camel	5	1–2	7
Mink	3	28–37	95
Goat	1	1	1
Fox	1	1	1
Dog	2	1	2
Arctic fox	2	5	6
Unidentified	6	–	15
Miscellaneous anthrax-positive samples†			
Soil samples	17	–	–
Wool	1	–	–

*0 indicates animals that recovered from infection.
†*Bacillus anthracis* spores were recovered, but there were no infections.

and MEGA 3.1 phylogenetic software programs. UPGMA cluster analysis of the Kazakh isolates with complete MLVA-8 data (*4*) identified 12 unique MLVA subtypes.

UPGMA cluster analysis of the 12 Kazak MLVA genotypes (G_{kz}) with the diverse 89 genotypes reported by Keim et al. (*4*) showed that most isolates (n = 78) belonged to the previously described A1.a genetic cluster; 6 isolates belonged to the A3.b cluster; and 2 isolates belonged to the A4 cluster. More than half of the A1.a isolates belong to previously described genotypes (38/74; excluding samples with missing pX01, pX02 data), including the previously described MLVA genotypes 3 (n = 15), 6 (n = 2) and 13 (n = 21). Most of the novel genotypes reported from the Kazakhstan National collection represent slight variants of previously described genotypes that can be explained by the insertion or deletion of ≥1 tandem repeats at a particular locus, usually in pX01 or pX02 (Table 3). However, 2 of the genotypes from Kazakhstan (G_{kz}-9 and -11) appear to represent new sublineages on the basis of newly described allele combinations and distance-based clustering with the diverse 89 genotypes. In addition, the pX01 allele sizing at position 138 appears novel (G_{kz}-5); we have not seen this size reported in previous MLVA-8 studies (Table 3).

Geographic Distribution of MLVA Genotypes

The geographic distribution of MLVA types in Kazakhstan indicated that A1.a genotypes were widely distributed (Figure 3). For example, the most common Kazakh genotype (G_{kz}-1; n = 21) clusters on the Georgia–Kazakhstan border and on the southern border near Kyrgyzstan and Uzbekistan. The A1.a G_{kz}-4 (n = 17) is also widely dispersed across Kazakhstan; cases have occurred in the western, southern, and eastern regions and into Kyrgyzstan. Specific genotypes within the Kazakh A1.a group appear to exhibit geographic clustering, reflecting temporally linked outbreaks.

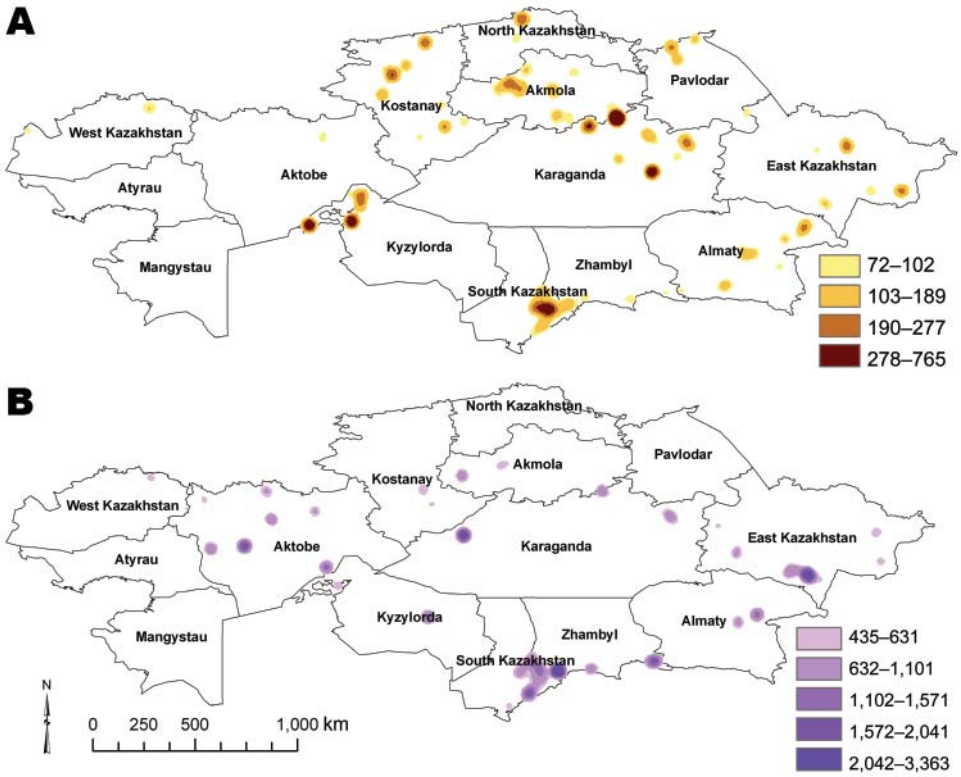

Figure 2. Kernel density estimates of anthrax outbreaks in cattle (A) and sheep (B), Kazakhstan, 1937–2005. Color shading represents SD values relative to density values from the kernel density estimate analysis for each species.

Table 3. Variable number tandem repeat sizes for *Bacillus anthracis* isolates, Kazakhstan*

Kazakhstan genotype no.	MLVA group†	MLVA genotype	*vrrA*	*vrrB1*	*vrrB2*	*vrrC1*	*vrrC2*	*CG-3*	*pX01*	*pX02*
1	A1.a	Gt-13 (*4*)	313	229	162	613	604	153	132	137
2	A1.a	Novel	313	229	162	613	604	153	135	137
3	A1.a	Novel	313	229	162	613	604	153	129	139
4	A1.a	Novel	313	229	162	613	604	153	129	137
5	A1.a	Novel	313	229	162	613	604	153	138	137
6	A1.a	Gt-6 (*4*)	301	229	162	613	604	153	126	137
7	A1.a	Gt-3 (*4*)	313	229	162	613	604	153	126	137
8	A1.a	Novel	313	229	162	613	604	153	132	139
9	Novel	Novel	325	229	162	613	604	158	132	137
10	A4	Novel	313	229	162	538	604	158	126	137
11	Novel	Novel	313	229	162	583	532	153	129	141
12	A3b	Novel	313	229	162	583	532	158	126	139

*Raw allele sizes were determined by electrophoresis on the ABI 310 (Applied Biosystems, Inc., Foster City, CA, USA); sizes were compared to control variable number tandem repeats and corrected to the sizes reported by Keim et al. (*4*).
†MLVA, multilocus variable number tandem repeat. MLVA group determined by unweighted pair group method arithmetic mean clustering with the diverse 89 genotypes described by Keim et al. (*4*).

The KZ genotypes 9–12 (G_{kz}-9–12) also appear to be more geographically confined, although this apparent confinement is likely a reflection of sample size. For example, isolates with G_{kz}-12 (n = 6; Figure 3) are exclusively found in the border region of the East Kazakhstan oblast, whereas the group 9 isolates (n = 5) are found in the Shymkent oblast in the south-central portion of the country. MLVA G_{kz}-11 (n = 1), which appears to represent a previously unreported genetic lineage, was isolated just south of Kazakhstan in Kyrgyzstan.

SNP Typing

Representative cultures from each of the Kazakh MLVA genotypes plus the Russian STI vaccine were SNP genotyped by using allelic discrimination probes and the Light Cycler II instrument. The SNP results were compared (Table 4) with the SNP profiles of Van Ert et al. (*10*), allowing assignment of the isolates to 1 of 12 sublineages. As with MLVA typing, all isolates tested with SNPs had genotypes characteristic of the A branches.

Representatives of MVLA genotypes 1–9 were assigned to A.Br.008/009, KZ genotype 10 to the A.Br.Vollum subgroup, and genotype 11 and 12 to the A.Br.Ames subgroup. The SNP data indicated that all representative A1.a Kazakh isolates belonged to the European branch of this group. The assignment of MLVA G_{kz}-10 to the A.Br. Vollum group is consistent with *B. anthracis* found globally in areas such as Pakistan and western China (*10*). Likewise, the assignment of Kazakh MLVA genotypes 11 and 12 to the A.Br.Ames genotype is consistent with the presence of this lineage in China (*10*).

Discussion

The historical occurrence and geographic distribution of anthrax outbreaks in Kazakhstan suggest anthrax foci are heavily concentrated in the southern region and broadly distributed across the northern portions of the country but are less common in the central regions. This may reflect regional differences in soil composition, availability of water and livestock and even case reporting. For example, the central region of Kazakhstan is dominated by desert, which likely has poor soils for long-term spore survival, whereas in the southern, northern, and eastern oblasts, the soils are more alkaline with higher organic matter and likely support spore survival (*15–17*). From a temporal perspective, outbreaks (or outbreak reports) have decreased in severity (number of animals infected), frequency (number of reported outbreaks), and have been associated with smaller geographic areas affected. However, the spatial distribution of the disease appeared to be relatively stable in the northern and southern Kazakh oblasts during the study period.

From a genetic perspective, *B. anthracis* in Kazakhstan was dominated by isolates clustering in the MLVA A1.a group, which is consistent with reports of the A1.a group being widely distributed globally (*4*,*5*,*6*). The widespread occurrence and apparent ecologic establishment of these VNTR genotypes in Kazakhstan supports the hypothesis that the A1.a group represents a very fit strain complex (*6*). Of the 8 A1.a genotypes in Kazakhstan, 5 were novel (G_{kz}-2, -3, -4, -5, and -8) and exhibited a previously undescribed pX01 allele (G_{kz}-5), which is not unexpected considering that this region has been underrepresented in prior MLVA-8 *B. anthracis* studies (*4–8*).

SNP typing of representative isolates from the A1.a Kazakh MLVA genotypes assigns these isolates to the A.Br.008/009 SNP lineage, which is widely distributed throughout Europe and has been reported in western China (*10*,*18*). Notably, the SNP data differentiate the Kazakh genotypes from the related North American genotypes, which are not effectively differentiated by MLVA alone. Since the representative Kazakh isolates in this SNP study were cultured from outbreaks spanning a 50-year

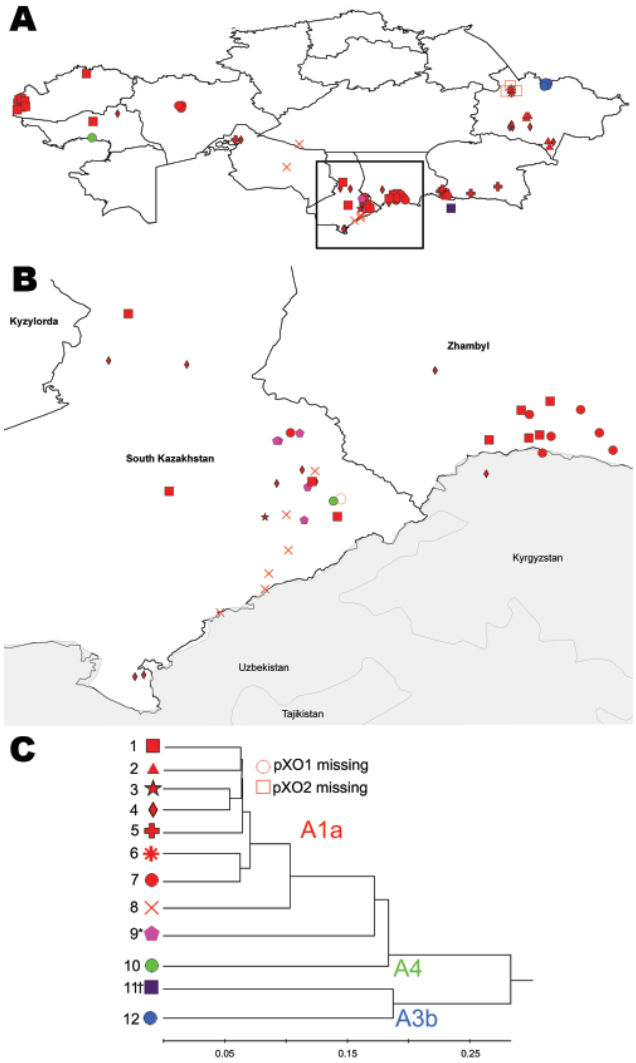

Figure 3. Geographic distribution of genotypes of *Bacillus anthracis* strains in Kazakhstan (A), with a closer view of outbreaks within eastern and southern Kazakhstan (B). Different genotypes are represented by different shapes and color coding reflecting major genetic affiliations (C). * and † indicate novel subgroups. Scale bar indicates genetic difference.

period (1952–2002), our data not only expand the understanding of the geographic range of this Eurasian lineage (A.Br.008/009) but also provide insights into its historical incidence and persistence in the country. Because of sampling limitations, the extent to which this dominant lineage is represented in the northern sections of Kazakhstan, and further into Russia, is unknown. However, in a recent study *B. anthracis* DNA from persons affected by the Sverdlovsk accident was assigned to the A.Br.008/009 SNP subgroup (*19*). Our data and the report that the Sverdlovsk strain was initially isolated in the 1950s in Kirov, Russia (*19*), underscores the need to genotype additional samples in northern Kazakhstan oblasts and Russia to measure the northern range of this apparently highly successful lineage.

The assignment of Kazakh isolates to the A3.b and A4 MLVA clades and the A.Br.Ames and A.Br.Vollum SNP groups is not surprising considering these MLVA and SNP types are also found in Middle Eastern countries, such as Pakistan and China (*10*). As first reported by Van Ert et al. (*10*), and later detailed by Simonson et al. (*18*), the A.Br.001/002 is common in China, whereas the closely related A.Br.Ames SNP lineage is more restricted geographically. The finding that the Kazakh isolates from the eastern border were assigned to A.Br.Ames SNP group is notable considering that the A.Br.Ames isolates that can be geolocated are found exclusively in Inner Mongolia. These genotypic similarities may reflect historical trade and nomadic routes linking those regions.

The absence of B lineage genotypes in Kazakhstan, as indicated by both MLVA and SNP data, is consistent with the lack of these genotypes in China, including the western province of Xinjiang (*10*,*18*), and supports the hypothesis that these lineages are restricted to narrow environmental conditions and, therefore, are more restricted in their global distribution (*9*). On a more local level, our MLVA data permit strain-level analysis of samples isolated during outbreaks. In several instances we were able to link strains collected from human anthrax patients to the infection source. For example, we identified the same strain in 10 cultures collected from an outbreak in western Kazakhstan that occurred from July–August 2005. The samples included cultures isolated from livestock, contaminated meat, human victims, and contaminated soil. The MLVA data linked the cultures and provided a mechanism for retrospective epidemiologic trace-back.

Sampling biases and limitations are important considerations in any study. For example, the distribution of cultures available for this study does not represent a balanced sampling of the entire country. There is an ongoing effort in Kazakhstan to expand the culture collection and to include a wider geographic sampling of the country, including the northern oblasts, which is underrepresented in the current culture collection but has a long historical record of anthrax. It would be worthwhile to revisit livestock burial sites and to isolate and analyze cultures from this region. In addition, the application of more comprehensive genetic analysis of Kazakh isolates would provide greater insight into the uniqueness of *B. anthracis* diversity in this region. For example, although canonical SNPs provide a powerful tool for assigning isolates into major clonal lineages, their resolution is limited by the use of relatively few representative SNPs and the diversity of the genomes used in the initial discovery process.

In summary, our work describes the historical incidence, distribution, and biochemical and genetic diversity

Table 4. *Bacillus anthracis* SNPs, Kazakhstan*

Isolate	KZ MLVA genotype	SNP group	SNPs											
			A branch								B branch			
			001	002	003	004	006	007	008	009	001	002	003	004
KZ 6	1	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 60	2	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 52	3	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 3	4	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 44	4	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 1	5	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 74	6	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 25	7	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 55	7	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 8	8	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 13	9	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T
KZ 11	10	A.Br.Vollum	T	G	A	T	A	C	T	A	T	G	G	T
KZ 42	11	A.Br.Ames	C	A	G	C	A	T	T	A	T	G	G	T
KZ 66	12	A.Br.Ames	C	A	G	C	A	T	T	A	T	G	G	T
KZ ST1	NA	A.Br.008/009	T	G	A	T	A	T	G	A	T	G	G	T

*SNP, single nucleotide polymorphism; KZ, Kazakhstan; MLVA, multilocus variable number tandem repeats; NA, not applicable. SNP changes are shaded. SNP groups as described in Van Ert et al. (*10*).

of *B. anthracis* isolates in the central Asian republic of Kazakhstan. Our discovery of novel genotypes in this region contributes to the understanding of the global diversity of the pathogen and emphasizes the need for future studies in this geographic region. In addition, this study provides useful baseline data for future epidemiologic studies in Kazakhstan and for guiding future disease control programs

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Dr Aikembayev is the director of the Infection Control Training Centre, Republican Sanitary Epidemiologic State, Almaty, Kazakhstan. His research interests include *B. anthracis* and *Yersinia pestis.*

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Methicillin-Resistant *Staphylococcus aureus* Carriage and Risk Factors for Skin Infections, Southwestern Alaska, USA

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Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) infections are common in southwestern Alaska. Outbreak strains have been shown to carry the genes for Panton-Valentine leukocidin (PVL). To determine if carriage of PVL-positive CA-MRSA increased the risk for subsequent soft tissue infection, we conducted a retrospective cohort study by reviewing the medical records of 316 persons for 3.6 years after their participation in a MRSA nasal colonization survey. Demographic, nasal carriage, and antimicrobial drug use data were analyzed for association with skin infection risk. Skin infections were more likely to develop in MRSA carriers than in methicillin-susceptible *S. aureus* carriers or noncarriers of *S. aureus* during the first follow-up year, but not in subsequent years. Repeated skin infections were more common among MRSA carriers. In an area where PVL-containing MRSA is prevalent, skin infection risk was increased among MRSA nasal carriers compared with methicillin-susceptible *S.aureus* carriers and noncarriers, but risk differential diminished over time.

Methicillin-resistant *Staphylococcus aureus* (MRSA) has become a primary cause of skin and soft tissue infections among persons without extensive exposure to healthcare settings. Nasal carriage of *S. aureus* is a known risk factor for these infections (*1*–*3*) and a common reservoir during skin and soft tissue infection outbreaks (*4*–*6*). Such outbreaks have occurred in community (*7*–*10*) settings, e.g., athletic team facilities (*11*–*16*), correctional facilities (*13*,*17*), and military basic training camps (*18*,*19*). Risk factors found in these community settings are frequent skin-to-skin contact (*11*,*12*,*14*,*15*), sharing of personal items without frequent cleaning (*11*–*14*), and MRSA carriage (*18*,*20*). Nasal colonization is also a risk factor for infections in hospital settings (*4*–*6*,*21*–*23*) and long-term care facilities (*24*,*25*). However, all of these previous studies have used a case–control study design, making it impossible to determine if MRSA carriage preceded infection.

Little is known about the subsequent risk for skin and soft tissue infections among persons colonized with MRSA. This lack of information becomes a question of clinical significance because increasing numbers of MRSA case-cluster investigations include nasal colonization studies that identify persons as MRSA-colonized. Clinicians and patients are left to consider whether interventions such as decolonization, continued monitoring, or restrictions in occupational activities are indicated. The notable absence of data regarding subsequent risk for illness among MRSA-colonized persons in community settings does little to inform these treatment decisions.

In southwestern Alaska, a dramatic increase in the number of skin infections led to an investigation by the Centers for Disease Control and Prevention and the Yukon Kuskokwim Health Corporation in 2000. MRSA exhibiting the type IV staphylococcal cassette chromosome *mec* gene had become the predominant community strain in that region, accounting for 100% of *S. aureus* isolates from skin infections

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(*7*,*8*). Also, the USA400 strain is the predominant strain of CA-MRSA in this area, whereas USA300 is the predominant strain in most other areas of the United States (*8*). In that initial investigation, we conducted a case–control study in 1 village in Alaska to assess risk factors for MRSA skin infections and evaluated nasal carriage among case–control participants and their household members (*1*). The present study is a follow-up assessment of participants in the prior nasal colonization survey. Our goal was to assess the risk for subsequent skin infections among persons whose nares cultures were colonized with MRSA (carriers) compared with those colonized with methicillin-susceptible *S. aureus* (MSSA), or those whose nares cultures were negative for *S. aureus* (non–*S. aureus* carriers).

Methods

This retrospective cohort study included persons who had been enrolled in a case–control study conducted in September 2000, which included anterior nares swab cultures for *S. aureus* obtained by using standard methods (*1*). The 316 study participants included 32 persons with a history of a culture-confirmed *S. aureus* skin infection (furunculosis or cellulitis) in the data collection period, 90 persons with no skin infection history in the year before the case–control study, and 194 household members of case- and control-patients. All persons were residents of 1 southwest Alaska village (population 713, accessible only by river or airplane) (*26*). Healthcare is provided by 1 integrated system, which includes a primary care clinic in the village and 1 regional hospital. The study was approved by the Institutional Review Board of the Centers for Disease Control and Prevention, area review boards in Alaska, and the local tribal health authority. A waiver of informed consent was obtained that enabled use of data from existing sources after delinking from individual identifiers.

Data Collection

We reviewed the medical records of all 316 persons to determine the total clinic or hospital visits by patients with skin infections or those for which an antimicrobial drug was prescribed during September 20, 2000–May 2, 2004 (3.6 years). Each participant's age, sex, *S. aureus* carriage status at the beginning of the study, and whether he or she lived with a MRSA carrier were recorded. We defined an antimicrobial drug course as a prescription for any oral or parenteral antimicrobial agent. Prescriptions involving simultaneous administration of >1 antimicrobial drug were counted as 1 course, as were changes in antimicrobial treatments for the same illness course that may have resulted from a participant's inadequate response to empiric therapy. For continuous prophylactic antimicrobial drugs, each month was counted as 1 course of treatment. Topical antimicrobial drugs were not counted.

Skin infections were defined as furuncles/abscesses, cellulitis, folliculitis, or deep wound infections as documented by the clinical provider. For each patient, we recorded visit date, diagnosis, anatomic site of infection, pathogen (if available), and consequent antimicrobial drug therapy. Visits for impetigo, scabies, and dermatitis were not included in the analysis. We considered skin infections to be distinct episodes if they occurred in the same anatomic site >6 months apart, and in different anatomic sites if they occurred >2 months apart. Only the first visit for treatment of a skin infection episode was recorded if there were multiple visits for the same episode. Nasal colonization was assessed only at the onset of the study. Isolates from skin infections of study participants were not available for comparison with nasal colonization specimens.

Statistical Analysis

Univariate analyses were performed to compare demographic and clinical characteristics among 3 carriage groups (MRSA, MSSA, and non–*S. aureus* infections). Demographic characteristics included age, sex, and *S. aureus* colonization status of household members. Clinical characteristics included the type and anatomic site of the skin infection, identified pathogens, and current history of antimicrobial drug treatment. Analyses were performed by using SAS version 8 software (SAS Institute, Inc., Cary, NC, USA). Univariate comparisons of categorical and continuous variables were conducted by using the χ^2 and Kruskal-Wallis tests, respectively. We assessed confounding of demographic characteristics through use of the Cochran-Mantel-Haenszel test. The Kaplan-Meier method was used to determine risk for first skin infection over time for each of the 3 groups. The log-rank statistic was used to compare survival (time without a skin infection) between carriage groups. Persons were censored at the time of death (n = 5) or at the time of last contact with the healthcare system for those who were known to have moved from the village (n = 3). We assessed confounding of other variables with a stratified nonparametric survival test (*27*).

Results

Study Population

Of the 316 participants, 41 (13%) were colonized with MRSA at the beginning of the study period (September 20, 2000); 85 (26.9%) were colonized with MSSA, and 190 (60.1%) were not colonized with *S. aureus*. Nasal carriage findings are described in more detail in a report of the prior case–control study (*1*). For the purposes of this study, 10 (24.4%) of those colonized with MRSA had been included as case-patients with a history of skin infections in the prior case–control study, 3 (7.3%) were control-patients with no history of skin infections in the previous year, and 28

(68.3%) were household members of case- or control-patients whose skin infection history before the study period was not obtained. Of those colonized with MSSA, 2 participants were case-patients, 28 were control-patients, and 55 were household members. Of the noncarriers, 20 were case-patients, 59 were control-patients, and 111 were household member participants. MRSA carriers were more likely to have a household member who was also a MRSA carrier (78%) compared with MSSA carriers (29%) and non–*S. aureus* carriers (17%) (p<0.0001). *S. aureus* carriers were, on average, younger (median 14 years) than non–*S. aureus* carriers (median 22 years, p = 0.02) (Table 1). However, MRSA carriers (median 13 years) and MSSA carriers (median 16 years) were of similar ages (p = 0.46).

Risk for Skin Infection

During the first year after the carriage study, ≥1 skin infections developed in 107 (33.9%) participants. Skin infections were more likely to develop in MRSA carriers (23/41 [56.1%]) compared with MSSA carriers (27/85 [31.8%], relative risk [RR] 1.76) and non–*S. aureus* carriers (57/190 [30.0%], RR 1.87, p = 0.005). For persons who did not have a skin infection diagnosed in the first year, the risk for developing a skin infection in the 2 to 3.6-year period did not differ significantly by carrier status: 5/18 (27.8%) of MRSA carriers, 20/58 (34.5%) of MSSA carriers, and 59/133 (44.4%) of noncarriers (p = 0.23). Over the entire 3.6-year follow-up period, ≥1 skin infections developed in a higher proportion of MRSA carriers (28/41 [68.3%]) than in MSSA carriers (47/85 [55.3%]) and non–*S. aureus* carriers (116/190 [61.1%]), but these differences were not statistically significant (p = 0.36). Rates for skin infections for the first 3 years of follow-up are shown in Table 2.

The median age of persons in which skin infections developed in the first year was 17 years and was similar for each carriage group (p = 0.50). Among persons in which skin infections developed during the full study period, the median age was similar for MRSA carriers (median 16.5 years) and MSSA carriers (median 14 years, p = 0.7), but was higher for non–*S. aureus* carriers (median 22 years, p = 0.06 vs. MRSA carriers).

The number of skin infections in the first year was higher for MRSA carriers (mean 0.7) than for either MSSA carriers (mean 0.4) or non–*S. aureus* carriers (mean 0.4, Table 2). For the entire follow-up period, MRSA carriers had a mean of 1.9 skin infections per person, which exceeded that of MSSA carriers (mean 1.2, p = 0.04) and the non–*S. aureus* carriers (mean 1.1, p = 0.02). No difference was detected in the numbers of skin infections by sex and carriage group in either analysis of the first year (p>0.3) or the full study period (p>0.9).

Among MRSA-colonized persons, skin infection risk did not differ by the colonization status of household members (p>0.22). However, among non–MRSA-colonized persons, skin infection risk in the first year was higher for those with a MRSA–colonized household member (23/57 [40%]) compared with those without a MRSA−colonized household member (61/208 [24%], RR 1.4, 95% confidence interval [CI] 1.0–2.1, p = 0.007); this difference did not persist when considering the entire follow-up period (p = 0.4). After adjusting for household member MRSA colonization, the RR for developing a skin infection in the first year was 1.6 (95% CI 1.1–2.4) when comparing MRSA carriers versus non–*S. aureus* carriers.

By using survival analysis, we found that after 1 year MRSA carriers were more likely than non–*S. aureus* carriers to have had a skin infection (RR 1.9, 95% CI 1.3–2.6, p = 0.001; Table 2). After 1 year, no difference was detected in the occurrence of a first skin infection between MSSA carriers and non–*S. aureus* carriers (p = 0.69). After 2 years, an increased risk remained between MRSA and non–*S. aureus* carriers (p = 0.02), but after 3 years this difference was no longer significant (Table 2). We stratified by gender, age, and household member colonization status; the results remained unchanged. The estimated length of time for 50% of the MRSA carriage group to have devel-

Table 1. Characterization of study participants by *Staphylococcus aureus* carriage group, Alaska, 2000*

Characteristic	MRSA carriers, n = 41	MSSA carriers, n = 85	Non–*S. aureus* carriers, n = 190	Total, n = 316	p value†
Male sex	23 (56.1)	40 (47.1)	94 (49.5)	157 (49.7)	0.6
Median age, y (range)	13 (0–77)	16 (1–73)	22 (0–85)	18 (0–85)	
Age group, y					
<5	10 (24.4)	17 (20.0)	29 (15.3)	56 (17.7)	0.0456
5–19	15 (36.6)	35 (41.2)	57 (30.0)	107 (33.9)	
20–39	9 (22.0)	20 (23.5)	60 (31.6)	89 (28.2)	
40–64	6 (14.6)	9 (10.6)	32 (16.8)	47 (14.9)	
>64	1 (2.4)	4 (4.7)	12 (6.3)	17 (5.4)	
Household member nasal carriage status					
≥1 MRSA+	32 (78.0)	25 (29.4)	32 (16.8)	89 (28.2)	<0.0001

*Values are no. (%) except as indicated. MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.
†χ^2 tests were used to detect differences in carriage groups by sex and household member status; nonparametric 1-way analysis of variance software (SAS Institute, Cary, NC, USA) was used to detect differences in carriage groups by age.

Table 2. *Staphylococcus aureus* skin infections among study participants, by year, Alaska, 2000–2004*

Skin infection outcome	MRSA carriers, n = 41	MSSA carriers, n = 85	Non–*S. aureus* carriers, n = 190	p value: MRSA vs. non–*S. aureus*	p value: MSSA vs. non–*S. aureus*
Skin infection rate, %					
Year 1	56	32	30	0.001	0.77
Year 2	37	26	30	0.42	0.50
Year 3	32	21	21	0.15	0.98
Cumulative % with ≥1 skin infection†					
Year 1	56	32	30	0.001	0.69
Year 2	61	41	48	0.02	0.48
Year 3	66	52	58	0.07	0.50
1st year of follow-up					
Mean no. skin infections‡	0.7	0.4	0.4	0.001	0.73
No. (%) with ≥2 infections§	6 (15)	7 (8)	13 (7)	0.10	0.68
Entire follow-up period, 3.6 y					
Mean no. skin infections‡	1.9	1.2	1.1	0.03	0.60
No. (%) with ≥3 infections§	13 (32)	13 (15)	24 (13)	0.02	0.72

*MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S. aureus*. Skin infection rate per 100 persons in each of the 3 years; 2-sample Poisson test was used to compare skin infection rates.
†Comparisons made by use of Log-Rank statistic in Kaplan-Meier estimation of survival curve.
‡Comparisons made by use of Kruskal-Wallis statistic.
§Chi-square test used for calculation of p value.

oped a skin infection was 289 days (0.8 years), 1,049 days (2.9 years) for MSSA carriers, and 804 days (2.2 years) for non–*S. aureus* carriers (Figure).

Skin Infection Characterization

A total of 391 skin infections occurred during the 3.6-year follow-up. Of these, 246 (62.9%) presented as a single furuncle; 85 (21.7%) were multiple furuncles; 50 (12.8%) had evidence of cellulitis; 4 (1.0%) were diagnosed as folliculitis; and 6 (1.5%) were deep abscesses. One hundred thirteen (45.9%) single boils occurred in the buttocks/low back/thigh area; 35 (41.2%) multiple boil infections occurred on the buttocks/low back/thigh area. Thirty-three (66.0%) of the cellulitis infections occurred on the extremities.

For all skin infections on which antimicrobial drug data were available, 264 of 382 (69.1%) patients were prescribed antimicrobial drugs. We found no difference in the mean number of antimicrobial drug treatment prescribed over the study period to *S. aureus* carriers compared with non–*S. aureus* carriers (12.0 vs. 10.4, respectively; p = 0.12) or for MRSA carriers (mean 13.6) as compared with MSSA carriers (mean 11.3, p = 0.17).

During the entire course of follow-up, 79/383 (21%) of infections (7 with an unknown culture status) were cultured. The proportion of skin infections cultured was similar for MRSA carriers (22%), MSSA carriers (22%), and non–*S. aureus* carriers (20%). In the 3.6 year follow-up period, 70 (89%) skin infections cultured were MRSA, 6 (8%) were MSSA, and 3 (4%) were other pathogens. Over the entire study period, 12/41 (29%) of MRSA carriers had a MRSA confirmed skin infection, compared with 12/85 (14%) of MSSA carriers and 30/190 (16%) of non–*S. aureus* carriers (p = 0.05 for MRSA vs. MSSA carriers, p = 0.004 for MRSA vs. non–*S. aureus* carriers, and p = 0.72 for MSSA vs. non–*S. aureus* carriers).

Discussion

In this study of a rural village in southwestern Alaska where MRSA was responsible for 86% of skin infections, we recruited a cohort of participants from a community setting to determine risk factors for the development of skin infections. We found nasal MRSA carriage to be a significant risk factor for skin infections in the first year when compared with MSSA and non–*S. aureus* carriers. More skin infection episodes also developed in these MRSA carriers than other carriage groups in the first year and entire 3.6-year follow-up period. We note that the risk for skin infections among MRSA carriers decreased with time but not for MSSA or non–*S. aureus* carriers; rates did not differ significantly between the 3 groups by the end of the study period. MRSA was the cause of 90% of skin infections in the follow-up period, with a similar proportion of cultures obtained among the carriage groups. Notably, skin infection took longer to develop in MSSA carriers, which may suggest that MSSA carriage provides some protection against MRSA infection. The strengths of this study are the long follow-up period, the single healthcare system that enabled capture of all clinic and hospital visits, and the location (an isolated community, which had recently experienced an outbreak of MRSA skin infections).

Having a household member who was a MRSA carrier was associated with an increased risk for skin infection in the first follow-up year for MSSA carriers and non–*S.*

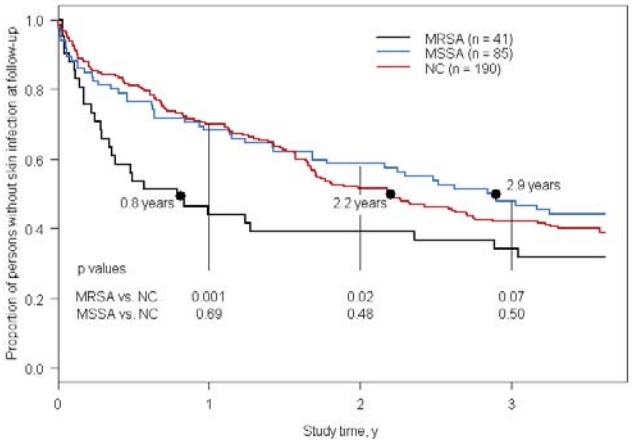

Figure. Kaplan-Meier survival curve of time until first skin infection among methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), and non–*S. aureus* carriers (NC). Black dots and associated text show the median time to first skin infection for each of the 3 groups.

aureus carriers but not for MRSA carriers. Studies by Boubaker et al. (*28*) and Osterlund et al. (*9*) have shown that household members of MRSA–colonized persons are at increased risk for becoming colonized. Our data support the hypothesis that transmission of MRSA from carriers to non-colonized household members is a risk factor for disease acquisition. Transmission of MRSA from household carriers during the follow-up period may also explain the continued risk for skin infections observed among MSSA and non–*S. aureus* carriers throughout the study. In contrast, the risk for skin infection among MRSA carriers was greatest in the first follow-up year but decreased thereafter, possibly indicating acquisition of immunity in this group. It is crucial to note that after the initial assessment the nasal carriage status of participants is unknown.

In the case–control study that preceded this investigation, antimicrobial drug use in the 12 months preceding the MRSA outbreak was associated with an increased risk for MRSA infection (*1*). However, in this study antimicrobial drug use did not differ among the 3 carriage groups and thus was not associated with subsequent risk for disease from MRSA. The small sample size of cultured skin infections and changes in clinical guidelines limit detection of any association. Antimicrobial drug use may still be a risk factor for MRSA infection in this community, although it was not demonstrated in this cohort. Alternatively, this finding may indicate a decreased role for antimicrobial drugs as a risk factor for MRSA infection once MRSA is established as a common colonizing organism in a community.

Our study population experienced a high incidence of skin infections compared with those in other published reports. In the first follow-up year, we found that 56% of MRSA carriers, 32% of MSSA carriers, and 30% of non–*S. aureus* carriers developed skin infections. A similar study by Muder et al. in a long-term care facility showed infection rates of 25%, 4%, and 4% for the same carriage groups, respectively, but patients were only followed while in the hospital and median duration of follow-up was <1 year for the carriage groups (*24*). Another prospective study of soldiers by Ellis et al. found that 38% of MRSA carriers, 3% of MSSA carriers, and 2% of non–*S. aureus* carriers developed subsequent skin infections (*18*). The higher incidence of skin infections may have been due, in part, to the absence of piped in-home water and wastewater service in this village. Lack of in-home running water has been associated with increased rates of skin and respiratory tract infections in rural Alaska, presumably due to decreased opportunities for hand and body hygiene. When household water must be carried into the home in buckets, residents may not wash their hands or bathe as often as they would if they had water available by turning a tap (*29*). The high skin infection rates could also be due to MRSA-colonized biofilms in saunas; 49% of saunas tested were positive for outbreak-strain MRSA. Sauna use >2 hours per week was reported by 68% of participants (*1*).

This study had several limitations. Persons with a history of frequent skin infections before the follow-up period may have been more likely to develop skin infections during the chart review period. However, we were not able to control for the potential confounder of past skin infections because of the limited dataset available on the cohort. Selective pressure for CA-MRSA carriage may have diminished during the follow-up period, because new antimicrobial drug treatment guidelines were implemented to reduce overtreatment with broad-spectrum antimicrobial drugs and first-generation cephalosporins as the outbreak progressed. Another limitation is that *S. aureus* nasal carriage status was assigned based on cultures performed at the beginning of the study period but colonization status was not assessed further; nasal carriage of *S. aureus* is known to be naturally transient in many carriers, or may also have been affected by use of antimicrobial drugs. Therefore, the effect of duration of carriage or crossover from 1 study group to another could not be determined. Study participants could have moved or been lost to follow-up, therefore these data represent minimal incidence estimates for the population. Another limitation is that behavioral data were not available for known risk factors for MRSA skin infections, such as sauna use or skin contact. MRSA carriage can occur in other body sites, such as the groin or axillae; our nasal carriage survey may have underestimated actual MRSA carriage.

Our study supports the hypothesis that nasal carriage of MRSA is a risk factor for skin infections, and that the risk may decrease over time relative to MSSA carriers and non–*S. aureus* carriers. We found that MRSA carriage

among household members increased the risk for skin infection among non–MRSA carriers. This information may be useful for education of persons identified as MRSA carriers or with MRSA–colonized household members to reinforce the value of hand hygiene practices and other measures that have been recommended to prevent the spread of MRSA within households. Further study of MRSA transmission in community settings is needed along with interventions that are designed to minimize pathogen transmission to close contacts and household members.

Ms Stevens is a fourth-year medical student at the University of Washington School of Medicine in Seattle. Her research interests include infectious diseases in rural underserved and international populations.

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Capacity of Public Health Surveillance to Comply with Revised International Health Regulations, USA

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Public health surveillance is essential for detecting and responding to infectious diseases and necessary for compliance with the revised International Health Regulations (IHR) 2005. To assess reporting capacities and compliance with IHR of all 50 states and Washington, DC, we sent a questionnaire to respective epidemiologists; 47 of 51 responded. Overall reporting capacity was high. Eighty-one percent of respondents reported being able to transmit notifications about unknown or unexpected events to the Centers for Disease Control and Prevention (CDC) daily. Additionally, 80% of respondents reported use of a risk assessment tool to determine whether CDC should be notified of possible public health emergencies. These findings suggest that most states have systems in place to ensure compliance with IHR. However, full state-level compliance will require additional efforts.

The 2005 revisions to the International Health Regulations (IHR 2005) were a major global policy achievement to ensure international recognition and notification of unusual public health events. These regulations are an international legal instrument that binds 194 countries (World Health Organization [WHO] member states). The goal of IHR 2005 is to help the international community prevent or respond to acute public health risks that have the potential to cross borders and threaten the global population. As seen with the emergence of pandemic (H1N1) 2009, diseases have the potential to spread quickly around the globe through international travel and trade (*1*). Member states are required to report certain diseases and public health events to WHO. Furthermore, the rights and obligations of member states are defined to establish procedures that WHO must follow to uphold global public health security (*2*).

The 2005 revision was the first major update to the IHR since 1969 and was designed to reflect trends in disease emergence and spread over the past several decades. The 2005 revision also was meant to unify the considerable changes in communication capacity, disease surveillance, and investigation infrastructure. Member states must report potential public health emergencies of international concern (PHEIC), including those of biologic, chemical, radionuclear, or unknown origin, to WHO. A common decision matrix that focuses national reporting around a risk assessment process is used rather than sole reliance on reporting of specific diseases or incidents (*3*). This method of reporting requires all member states to develop, strengthen, and maintain a core set of public health surveillance and response capacities at the local, intermediate, and national levels (*4*).

After IHR revision in 2005, member states were provided a 2-year window in which to assess their surveillance and response capacities, focusing on 4 necessary characteristics of surveillance systems: timeliness, sensitivity, stability, and usefulness (*3*). Each of the 194 member states also was required to designate a National Focal Point that would assess any event within 48 hours. After the assessment specified in Appendix 2 of IHR 2005, each member state must notify WHO of any potential PHEIC. Therefore, core public health surveillance systems at local and national levels must be capable of ensuring national awareness of incidents in a timely manner.

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Surveillance systems exist at many levels: clinics; hospitals; and local, state, national, regional, and global levels. To be effective, these different levels must be well integrated. Literature on surveillance systems often examines each level separately or, at most, the links between 2 adjacent levels, describing the importance of integrating each system so that communication between levels is more effective (*3*,*5*–*7*). State-to-national notification is a key aspect of federalist systems and has been viewed as a key challenge for countries with this type of government structure (*3*); several authors have noted the political and practical difficulties these surveillance systems may face and the various ways national disease surveillance can be facilitated (*8*).

In the United States, reporting of nationally notifiable diseases to the Centers for Disease Control and Prevention (CDC) by states is voluntary. Public health surveillance takes place within a state on the basis of reports received from a variety of sources, typically local. Reporting is mandated by state legislation or regulation. States then determine whether CDC should be notified. Notifications from states, territories, and the District of Columbia are collected and analyzed by the National Notifiable Diseases Surveillance System. A 2004 review of this system showed that for meningococcal disease laboratory results, local entities reported to their states and then states notified NNDSS within 2–117 days (*5*). More recently, 60% of meningococcal diagnosis reports were received by states within 1 day after diagnosis (*9*).

The literature identifies essential elements that surveillance systems need to meet IHR 2005 criteria, including electronic information systems and supportive infrastructure, to ensure timely reporting to the National Focal Point (*6*,*7*). In addition, intergovernment cooperation with both formal and informal communication, from the local to the international level, are essential aspects of successfully functioning public health surveillance systems (*3*,*6*,*8*) and can ensure rapid reporting of incidents before laboratory confirmation is received (*3*,*6*). Overall, key aspects of successful surveillance systems identified in the literature align closely with the requirements of IHR 2005. This alignment suggests that systems built in accordance with the agreement will provide successful global coverage.

Since the new regulations took effect, no studies have been done to determine the timeliness of reporting conditions specified in IHR 2005. In addition, no reports exist that discuss timeliness of notification to CDC about unusual cases or outbreaks of unknown cause. To address these gaps and to determine the ability of states to comply with IHR 2005, we assessed state surveillance capacities through surveys completed by the Council of State and Territorial Epidemiologists (CSTE). This assessment examined several key requirements that are necessary to effectively meet national responsibilities and ensure compliance with IHR 2005.

Methods

In February 2009, CSTE electronically distributed a structured, self-administered questionnaire to state epidemiologists in all 50 states and Washington, DC. Responses were made anonymous at the time of data analysis. The questionnaire was designed to address the following questions: 1) How are states able to determine the status of potential public health emergencies? 2) Are local health departments able to report in a timely manner to the correct point-of-contact (POC) in their state? 3) Are states able to notify CDC of public health emergencies in a timely manner (i.e., within 24 hours)? 4) Do states support or implement other control measures (i.e., collaboration with other departments or cross-jurisdiction)? Frequencies and percentages were used to describe the results.

The CSTE State Reportable Conditions Assessment, completed by state epidemiologists, also was used to assess state reporting practices. Information was obtained from the 2007 Assessment, which is available for review by all states and territories (*10*).

Results

A total of 47 (92%) of the 51 eligible jurisdictions responded to the questionnaire. Eighty percent of respondents reported the use of risk assessments to determine the necessity of notifying CDC about unusual or unexpected events (Figure 1). Of those who used risk assessments, ≈50% used them to initiate formal investigations. About 50% of respondents reported the use of risk assessments to evaluate whether notification to the state health officer (51%), chief emergency response/management office (47%), and CDC or other federal entities (51%), respectively, was necessary. Twenty-eight percent reported use of a state-based algorithm; 25%

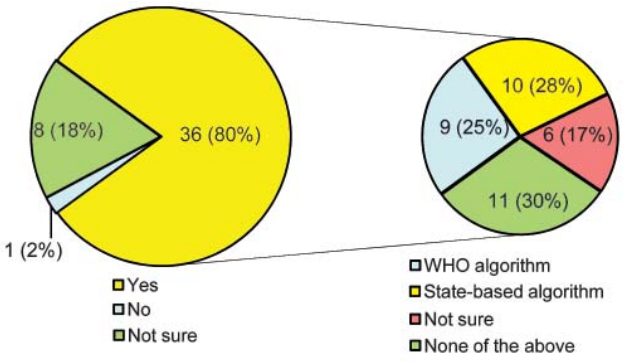

Figure 1. Proportion of state epidemiologists who use risk assessments to determine whether notification to the Centers for Disease Control and Prevention is necessary, showing types of algorithms used, United States, 2009. WHO, World Health Organization.

reported use of the WHO algorithm for risk assessments. The remainder of respondents used another algorithm (excluding a state-based algorithm or the WHO algorithm) or were not sure of the algorithm used in their state.

More than 90% of jurisdictions required reports of suspected and probable cases of the 4 immediately notifiable IHR 2005 conditions (i.e., smallpox, poliomyelitis caused by wild-type poliovirus, human influenza caused by a new subtype, and severe acute respiratory syndrome [SARS]) within 24 hours after diagnosis (Table 1). In addition, 96% of states reported that they would notify CDC of suspected and probable cases of IHR 2005 conditions within 24 hours (Table 1). Eighty-one percent of respondents reported having the capacity to transmit daily notifications to CDC. Of those unable to transmit daily notifications, 5 indicated that they would be able to provide daily electronic data to CDC within ≤1 year

All respondents reported they would either always or sometimes notify CDC of an unusual or unexpected case or outbreak; 60% reported they would always notify CDC

Table 1. Reported circumstances and time frames for reporting and notification of International Health Regulations conditions by state epidemiologists, USA, 2009*

Circumstance	Immediately, no. (%)	Within 4 h, no. (%)	Same business day, no. (%)	Within 24 h, no. (%)	Total no.
Novel influenza virus					
Time frame for reporting					
Suspected	23 (52)	2 (5)	1 (2)	5 (11)	37
Probable	1 (2)	0	0	1 (2)	4
Total†	25	2	1	6	44
Time frame for notification					
Suspected	16 (36)	9 (20)	3 (7)	1 (2)	29
Probable	9 (20)	0	3 (7)	1 (2)	14
Confirmed	0	0	1 (2)	0	1
Total†	26	9	7	2	45
Severe acute respiratory syndrome					
Time frame for reporting					
Suspected	25 (56)	1 (2)	4 (9)	8 (18)	41
Probable	1 (2)	1 (2)	0	0	2
Confirmed	0	0	0	1 (2)	1
Total†	27	2	4	9	45
Time frame for notification					
Suspected	16 (36)	9 (20)	4 (9)	2 (4)	32
Probable	8 (18)	1 (2)	3 (7)	0	12
Confirmed	1 (2)	0	0	0	1
Total†	25	10	7	2	45
Smallpox					
Time frame for reporting					
Suspected	30 (67)	2 (4)	1 (2)	6 (13)	41
Probable	0	1 (2)	0	0	2
Confirmed	0	0	0	1 (2)	1
Total†	31	3	1	7	45
Time frame for notification					
Suspected	23 (51)	5 (11)	4 (9)	1 (2)	33
Probable	8 (18)	2 (4)	0	0	11
Total†	32	7	4	1	45
Poliomyelitis, wild type					
Time frame for reporting					
Suspected	22 (49)	1 (2)	1 (2)	9 (20)	37
Probable	2 (4)	2 (4)	0	0	4
Confirmed	0	0	0	1 (2)	3
Total†	25	3	1	10	45
Time frame for notification					
Suspected	11 (24)	9 (20)	5 (11)	1 (2)	27
Probable	8 (18)	1 (2)	3 (7)	2 (4)	14
Confirmed	1 (2)	0	0	1 (2)	3
Total†	21	10	8	4	45

*n = 45 for all percentages except time frame for reporting of novel influenza virus (n = 44).
†Includes states that did not have a specified time for reporting and/or notification or they were not sure of the time frame for reporting and/or notification.

within 24 hours (Figure 2). Among the respondents, 30% would sometimes notify CDC within 24 hours. According to the 2007 State Reportable Conditions Assessment, 48 of the jurisdictions included unusual or unexpected events on their reportable conditions lists.

Furthermore, most states could identify a specific POC in their state for reporting various public health events and emergencies (Figure 3). Ninety-one percent of states reported having a designated POC for zoonotic, foodborne, and infectious events; for chemical and radiologic events, 84% and 86% of states, respectively, reported having a designated POC.

More than 50% of respondents reported having formal information-sharing systems or mechanisms pertaining to emergencies or outbreaks with state law enforcement, emergency management and homeland security, agriculture, environmental protection, and fish and wildlife agencies, excluding the state departments of transportation (37%) (Table 2). Fifty-one percent reported participating in cross-jurisdiction electronic surveillance and having reporting systems for foodborne and infectious diseases with neighboring states.

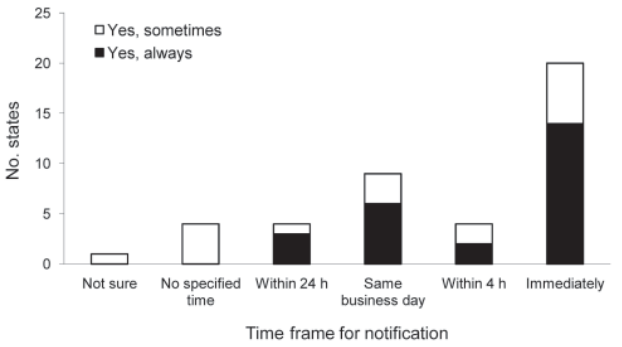

Figure 2. Number of states that notify the Centers for Disease Control and Prevention of an unusual or unexpected case or outbreak of disease, by time frame, United States, 2009.

Discussion

Most states can successfully conduct public health surveillance in compliance with IHR 2005. However, full state-level capacity for compliance was not found on any assessment response. Additional efforts are needed to ensure the ability of the United States to meet its IHR 2005 obligations.

Most states reported use of risk assessments to determine the need to notify CDC about unusual or unexpected events. In addition, ≈50% of states reported the use of risk assessment when initiating a formal investigation. IHR 2005 emphasizes the use of risk assessments to notify WHO about public health emergencies, rather than about specific events. The use of risk assessments in notifying CDC can help determine whether notification is necessary and ensure timely notification without waiting for laboratory confirmation.

Most (>80%) states reported having 1 POC for reporting chemical, radiologic, foodborne, infectious, and zoonotic events. All states should have a POC to facilitate prompt assessment and appropriate reporting. Such contacts also can assist in assessing events outside their areas of expertise or with unknown cause.

Reports of capacity to transmit daily notifications to CDC suggest that data collection and transmission capacity has improved substantially in recent years. The ability to transmit reports to CDC is a critical function, which allows for national situational awareness in high-profile events and public health emergencies.

Not all respondents included unusual or unexpected events on their state's reportable conditions lists. Adding this criterion would help ensure that conditions having the potential to become public health emergencies can be recognized and reported in a timely manner.

Internal and external relationships of each state can play a role in reporting. Most states have formal information-sharing systems with other agencies within their state. Such dissemination of information within a state increases the likelihood that IHR reportable events are appropriately evaluated and reported. Other agencies may have knowledge that could be incorporated into a risk assessment.

About 50% of respondents reported participation in cross-jurisdictional electronic surveillance and having reporting systems for foodborne and infectious diseases. Public health surveillance across state jurisdictions is as important as the sharing of information within a state. Neighboring states can be alerted to public health emergencies that have the potential to become widespread or even global.

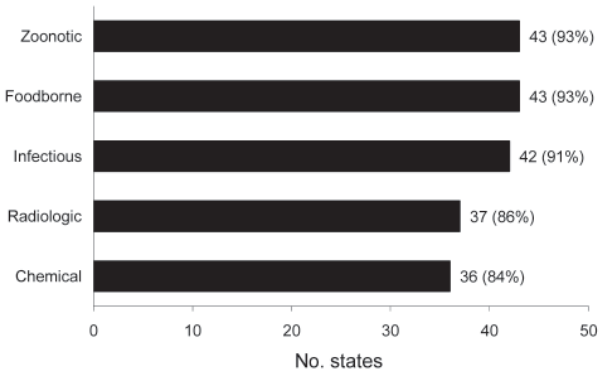

Figure 3. Number of state epidemiologists who have points-of-contact within the state for reporting different types of potential public health emergencies of international concern, United States, 2009.

Table 2. Proportion of state agencies that have formal information-sharing systems or mechanism for emergencies or outbreaks within state government, USA, 2009

Government agency	No./total reporting (%)
State law enforcement	33/44 (75)
Emergency management and/or homeland security	37/44 (84)
Agriculture	35/45 (78)
Transportation	17/44 (37)
Environmental protection	30/44 (68)
Fish and wildlife	28/45 (62)

Our assessment has several limitations. First, only states were assessed; neither territories nor local health departments were included. Circumstances are sufficiently different in the territories; no conclusions about their capacity should be drawn because they are likely to have different reporting practices and capacities. Future assessments should focus on identifying and documenting capacities for IHR 2005 compliance in at least a sample of jurisdictions at the local or county level. Second, our assessment relied on self-reporting, which in some instances may have led to inaccuracies or bias. Data were made anonymous before analysis so that states would not be singled out as having suboptimal reporting practices or capacities. Therefore, we could not inquire about potential discrepancies.

To enable the United States to fully meet its IHR 2005 obligations, all states should include unusual or unexpected events or outbreaks on their state lists of reportable conditions. In addition, states and CDC should work toward further development of the nationally notifiable conditions list and the timeframes for reporting. The capacity to transmit records to CDC on a daily basis is key to full compliance with IHR 2005. Risk assessments of unusual or unexpected events should be performed to determine whether they meet requirements for notification to CDC as a potential PHEIC. Performing such risk assessments will enable timely notification to CDC, even before laboratory confirmation. Furthermore, state POCs are likely to facilitate recognition and reporting of potential public health emergencies within their respective states.

State health departments should work to ensure that their counterparts in state government and in local health departments understand the requirements of IHR 2005; reporting exercises may help accomplish this goal. All health officials, particularly those at the state level, should have a basic understanding of these international regulations, especially the reporting and notification timeframes and practices. Reporting exercises would give state and local health officials the ability to assess potential public health emergencies in a practice environment and allow a broader perspective of when notification is necessary.

Expansion of cross-jurisdictional surveillance and reporting systems also would benefit national recognition and investigation of public health emergencies, especially for foodborne illness and infectious diseases. Such systems are not explicitly required by IHR 2005 but would assist states in the assessment and timely reporting of public health emergencies, both of which are necessary for compliance. National, state, and local government agencies should assist states in implementing these practices and developing appropriate infrastructures.

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Influenza Outbreaks during World Youth Day 2008 Mass Gathering

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Influenza outbreaks during mass gatherings have been rarely described, and detailed virologic assessment is lacking. An influenza outbreak occurred during World Youth Day in Sydney, Australia, July 2008 (WYD2008). We assessed epidemiologic data and respiratory samples collected from attendees who sought treatment for influenza-like illness at emergency clinics in Sydney during this outbreak. Isolated influenza viruses were compared with seasonal influenza viruses from the 2008 influenza season. From 100 infected attendees, numerous strains were identified: oseltamivir-resistant influenza A (H1N1) viruses, oseltamivir-sensitive influenza A (H1N1) viruses, influenza A (H3N2) viruses, and strains from both influenza B lineages (B/Florida/4/2006-like and B/Malaysia/2506/2004-like). Novel viruses were introduced, and pre-WYD2008 seasonal viruses were amplified. Viruses isolated at mass gatherings can have substantial, complex, and unpredictable effects on community influenza activity. Greater flexibility by public health authorities and hospitals is required to appropriately manage and contain these outbreaks.

Influenza is caused by a highly infectious respiratory virus with the potential to rapidly spread in susceptible hosts. Influenza outbreaks have frequently been described in populations such as residents of nursing care facilities (*1*,*2*), residential schools or colleges (*3*,*4*), prisons (*5*), military facilities (*6*), and other enclosed communities (*7*).

Mass gatherings pose complex and unique challenges to public health and medical services. Because populations are increasingly mobile, and more able to attend large gatherings, the risk for outbreaks of influenza and other infectious diseases among a susceptible population has increased, and a substantial responsibility is placed on health services if outbreaks occur. Despite this situation, influenza outbreaks during mass gatherings have rarely been described (*8*–*10*), and reports have not included results of detailed virologic testing. Furthermore, the effects of outbreak strains on local influenza epidemiology have not been assessed.

During World Youth Day (WYD2008) celebrations, 223,000 predominately young pilgrims from 170 countries attended a series of mass religious gatherings from July 15 to July 20, 2008, in Sydney, New South Wales (NSW), Australia. At the end of the week, an outdoor evening vigil with >200,000 participants preceded the final mass presided over by Pope Benedict XVI. This mass was attended by an estimated 400,000 persons (*11*). Approximately 100,000 pilgrims were given accommodation in sporting facilities, schools, and community centers, where temporary floor mats and blankets were provided and other facilities were shared. The largest site of accommodation, the Sydney Olympic Park site, provided lodging for as many as 12,000 pilgrims each night. Pilgrims remained housed overnight at the allocated accommodation sites and attended numerous outdoor religious gatherings during the day with other pilgrims. Many pilgrims traveled in Australia and New Zea-

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land before and after the WYD2008 celebrations, visiting major cities and rural areas.

We describe the epidemiologic and virologic features of an influenza outbreak predominantly among young adults during WYD2008 celebrations. These data provide insight into the complexity of influenza outbreaks during mass gatherings and their effects on the community at large. The insights gained should guide plans for mass events, particularly when held during periods of peak influenza activity.

Methods

Influenza was first identified among WYD2008 pilgrims on July 16, 2008. Emergency clinics were then quickly established to identify and isolate infected pilgrims. Symptomatic pilgrims were encouraged to visit the clinics, which were open 24 hours a day. Epidemiologic data were collected prospectively from all pilgrims who sought treatment. Respiratory tract samples (paired nose and throat swabs specimens) were obtained as previously described (*12*).

Influenza testing included the following: 1) point-of-care tests (POCTs) performed either on site or in the laboratory (Quickvue A & B; Quidel, San Diego, CA, USA, or BinaxNOW Influenza A & B, Binax, Scarborough, ME, USA), 2) antigen detection using type-specific indirect fluorescent antibodies (IFA) (Chemicon, Millipore, Billerica, MA, USA, or Bartels, Immunodiagnostic Supplies Inc., Bellevue, WA, USA); 3) validated in-house type- and subtype-specific nucleic acid testing (NAT) by using PCR that targeted the matrix, nonstructural, and hemagglutinin region of the influenza virus genome; and 4) virus culture using MDCK cells (*13*). IFA and NAT were the preferred diagnostic methods. The decision to perform POCT was made on a case-by-case basis by clinicians. Viral culture was performed on antigen- (POCT/IFA) or NAT-positive specimens. Testing was performed at 2 virology laboratories (Institute of Clinical Pathology and Medical Research, Westmead Hospital, Sydney, New South Wales, Australia; South Eastern Area Laboratory Service, Prince of Wales Hospital, Randwick, New South Wales).

Pilgrims with clinical or laboratory-confirmed influenza who sought treatment within 48 hours of symptom onset were offered oseltamivir (75 mg 2×/d for 5 days). Public health authorities recommended that infected pilgrims remain in isolation for 48 hours or until 5 days after symptom onset.

All influenza isolates from WYD2008 were sent to the WHO Collaborating Centre for Reference and Research on Influenza in Melbourne, Victoria, Australia, where antigenic analysis was performed by using a hemagglutination-inhibition assay (*14*). Oseltamivir susceptibility was tested by using a fluorescence-based neuraminidase (NA) inhibition assay (*15*). When no isolate was available, clinical samples were directly tested by rolling circle amplification for the H274Y mutation (the most frequently reported mutation conferring oseltamivir resistance) (*16*). Hemagglutinin (HA) and NA gene sequencing was performed by using standard methods (*17*). Sequence alignment was performed by using ClustalW (www.ebi.ac.uk/Tools/clustalw2/index.html) in DNAstar Lasergene version 8 (www.dnastar.com), and phylogenetic trees were generated by using maximum-likelihood (DNAML) in PHYLIP (*18*).

Australia-wide laboratory-confirmed influenza data were obtained from the National Notifiable Diseases Surveillance System (*19*). The influenza viruses isolated during WYD2008 were compared with viruses submitted to the WHO Collaborating Centre from all Australian states and territories during the 2008 influenza season. HA and NA sequences from WYD2008 viruses were compared with a representative sample of seasonal influenza viruses from around the world, including Australia, sequenced by the WHO Collaborating Centre.

Results

Respiratory tract samples were obtained from 227 WYD2008 attendees who sought treatment at established clinics. The true extent of infection is unknown because the pilgrims voluntarily visited the clinics, and respiratory tract sampling was limited at several accommodation sites after the outbreak was identified. The median age of the pilgrims tested was 21 years (range 12–72 years, interquartile range 18–28 years); 62.8% were female. Twenty-nine percent of pilgrims tested lived in Australia, and the remainder were from overseas (Europe, 28.0%; Oceania, 20.2%; North America, 17.1%; South or Central America, 2.6%; Asia, 2.6%; Africa, 0.5%). Recent influenza vaccination was infrequent; 25 (21.6%) of 116 reported recent vaccination (Southern Hemisphere, 12.0%; Northern Hemisphere, 27.9%; $p = 0.021$). Demographic characteristics of pilgrims who visited established clinics were not significantly different from those of the total pilgrim population (*11*).

Two or more influenza diagnostic tests were performed on all specimens (POCT, 80%; IFA, 100%; NAT, 97%; and virus culture, 43%). Laboratory confirmation of influenza virus infection was obtained for 100 (44.1%) pilgrims. This included 69 patients whose test results were positive by both antigen detection (POCT or IFA) and NAT, 21 patients with positive results by NAT yet negative by antigen detection, 5 patients with positive results by both antigen detection and viral culture, and 5 patients with positive results by 2 antigen detection methods (when clinical material was not sufficient for NAT or culture). Pilgrims had symptoms for a median of 2 days before they visited a clinic (95% confidence interval 1.7–2.7 days). No significant differences in age, sex, or country of origin were noted between the pilgrims with laboratory-confirmed cas-

es, pilgrims whose test results were negative, and pilgrims who were not tested (data not shown).

Influenza types A and B were identified during WYD2008 (Table). Influenza A was most frequently isolated from patients from Australia and Germany, whereas influenza B was most frequently isolated from patients from the Solomon Islands, Papua New Guinea, Australia, and North America. Numerous distinct circulating influenza viruses were identified: oseltamivir-resistant influenza A (H1N1) A/Brisbane/59/2007-like viruses, oseltamivir-sensitive influenza A (H1N1) viruses (no isolate was recovered for serotyping), influenza A (H3N2) A/Brisbane/10/2007-like viruses, and both influenza B viral lineages (B/Florida/4/2006-like [B/Yamagata-lineage] and B/Malaysia/2506/2004-like [B/Victoria-lineage] viruses).

This outbreak occurred in the context of low seasonal influenza activity in Australia (Figure 1). An increase in influenza A and B activity was identified in all Australian states and territories in the weeks after WYD2008. Influenza isolates from WYD2008 were compared with a representative sample of national seasonal influenza isolates (12.7% of the total Australian 2008 laboratory-confirmed influenza cases). Before WYD2008, influenza (H3N2) A/Brisbane/10/2007-like and B/Florida/4/2006-like viruses were the predominant early season viral subtypes/strains observed Australia-wide (33.3% and 58.8%, respectively; Figure 2). Subsequent to WYD2008, B/Malaysia/2506/2004-like and B/Florida/4/2006-like viruses were the most frequently identified influenza strains (41.0% and 35.7%, respectively).

The genetic relatedness of WYD2008 viruses to pre- and post-WYD2008 viruses was examined by sequence alignment of the HA gene (Figure 3) and NA gene (data not shown).

Before WYD2008, oseltamivir-resistant influenza A (H1N1) Brisbane/59/2007-like viruses were uncommon in Australia, and only 3 cases had been identified (Figure 2). Two cases were observed in South Australia 5 weeks and 3 days before WYD2008 (Figure 3, panel A, isolates 1–2). The remaining case was identified in Victoria in a returned traveler 5 days before WYD2008 (Figure 3, panel A, isolate 3). No obvious epidemiologic links were identified between pre-WYD2008 and WYD2008 oseltamivir-resistant influenza A (H1N1) viruses. Furthermore, isolates 1–3 appeared distinct from WYD2008 isolates. All WYD2008 influenza A (H1N1) isolates clustered relatively closely (Figure 3, panel A, isolates 4–16) and shared sequence homology with other 2008 Southern Hemisphere oseltamivir-resistant isolates. Epidemiologic data showed that most Australian and European pilgrims infected with oseltamivir-resistant A (H1N1) had traveled from Victoria to Sydney in the week before WYD2008. Despite this, oseltamivir-resistant A (H1N1) viruses were responsible for <10% of typed influenza virus infections. (Figure 2; Figure 3, panel A, isolates 17–19).

Genetic analysis of WYD2008 influenza A (H3N2) A/Brisbane/10/2007–like viruses demonstrated 2 distinct phylogenetic groups. The first influenza A (H3N2) cluster was obtained from Australian and New Zealand pilgrims only and was genetically related to sporadic Australian pre- and post-WYD2008 influenza isolates (Figure 3, panel B: pre-WYD2008, isolates 1–6; WYD2008 Australian/NZ cluster, isolates 7–8; post-WYD2008, isolates 9–13). The second phylogenetic group included influenza A (H3N2) viruses isolated from pilgrims from Germany and Italy (Figure 3, panel B: isolates 14–17; WYD2008 European cluster) and appeared to be closely related to influenza strains found in Southeast Asia in 2008. No evi-

Table. Virologic data from laboratory-confirmed World Youth Day 2008 influenza cases*

	Pilgrim country of origin, no. infections					
Virus type/subtype	Australia	Europe	Oceania	North America	Other region or unknown	Total no. infections
Influenza A (H1N1) (A/Brisbane/59/2007–like) oseltamivir resistant	14	6 (Germany, Czech Republic, France, Italy, Poland)	0	0	4	24
Influenza A (H1N1) (serotype unknown) oseltamivir sensitive	1	9 (Germany, Italy)	0	0	3	13
Influenza A (H3N2) (A/Brisbane/10/2007–like)	2	9 (Germany, Italy, Slovakia, Spain)	0	2 (USA)	1	14
Influenza A (subtype/serotype not available)	2	4 (Germany, France)	0	2 (USA)	2	10
Influenza B (B/Malaysia/2506/2004–like)	2	0	7 (Solomon Islands, New Zealand, Tonga, PNG)	3 (USA, Canada)	1	13
Influenza B (B/Florida/4/2006–like)	2	0	0	1 (USA)	1	4
Influenza B (serotype not available)	6	0	7 (Solomon Islands, PNG)	3 (USA, Canada)	6	22
Total	29	28	14	11	18	100

*PNG, Papua New Guinea.

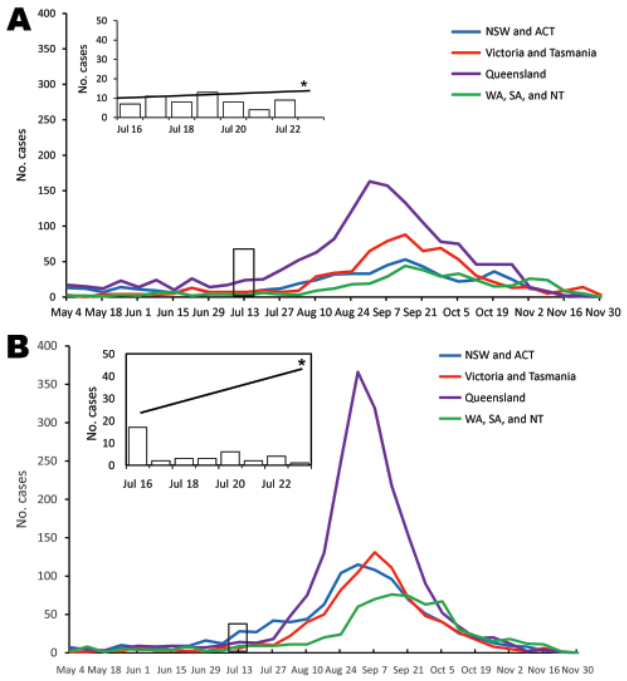

Figure 1. Laboratory-confirmed influenza A (A) and B (B) cases during World Youth Day 2008 (WYD2008; insets) compared with national seasonal influenza data (main graphs). Data are presented as the number of laboratory-confirmed cases per day for WYD2008 and per week for national influenza surveillance. Because laboratory methods to detect community influenza activity vary between different states, the relative effects of influenza in each state are not comparable. NSW, New South Wales; ACT, Australian Capital Territory; WA, Western Australia; SA, South Australia; NT, Northern Territory. *Background rate of laboratory-confirmed influenza for NSW/ACT included for comparison. †National data are inclusive of influenza cases diagnosed by antigen detection, nucleic acid testing, and viral isolation.

dence of similar strains was found in Australia before or after WYD2008.

Before WYD2008, only 4 cases of influenza B/Malaysia–like virus infection were identified Australia-wide (Figure 2). Two isolates from NSW were found to be distinct from WYD2008 isolates by sequence analysis (Figure 3, panel C, isolates 1–2). The remaining viruses (Figure 3, panel C, isolates 3–4) were identified in Queensland 2 and 5 days before WYD2008 and appeared to be closely related to WYD2008 isolates (Figure 3, panel C, isolates 5–10). No epidemiologic links to WYD2008 cases were identified. Pilgrims from the Solomon Islands sought treatment for a febrile illness at rural medical facilities 5 days before WYD2008, while they were staying close to the NSW/Queensland border. Subsequent review suggests that these illnesses were most likely influenza. WYD2008 pilgrims with confirmed influenza B/Malaysia–like virus infection were identified among contacts of these original clinical case-patients. Sequence analysis of WYD2008 isolates (Figure 3, panel C, isolates 5–10) clustered closely with isolates obtained throughout Australia post-WYD2008 (Figure 3, panel C: isolates 11–17). Because several pilgrims infected with influenza B/Malaysia-like viruses either lived or traveled through New Zealand en route to Sydney, we explored the idea that the influenza B/Malaysia from New Zealand was introduced. Peak influenza B/Malaysia activity in New Zealand occurred 4 weeks before peak activity was detected in Australia (*20*). Furthermore, WYD2008 isolates clustered closely with pre- and post-WYD2008 New Zealand isolates (Figure 3, panel C). Influenza B/Malaysia-like viruses also could have been introduced into both New Zealand and Australia from Pacific Island countries.No clear trends were identified with oseltamivir-sensitive influenza A (H1N1) and influenza B/Florida/4/2006-like viruses when WYD2008 and community isolates were compared (data not shown).

Discussion

Thousands of mass gathering events are held each year, including major sporting events, festivals, demonstrations, and pilgrimages. Mass gatherings of a scale seen with WYD2008 have the potential to create health risks for those attending and the community at large. Although outbreaks of communicable diseases during mass gatherings have been described, insufficient epidemiologic and pathogen-related data have been described to characterize the outbreak, measure the impact on the wider community, or guide management of future mass gatherings (*8–10*). WYD2008 presented a unique opportunity to study the effects of influenza on mass gatherings by combining epidemiologic data acquired through prospective data collection, laboratory data obtained after respiratory tract sampling, and surveillance data obtained through established Australia-wide influenza laboratory networks.

A notable influenza outbreak occurred during WYD2008, likely exacerbated by crowded living conditions, the presence of multiple circulating influenza viruses, and low immunization rates. The true effects of influenza infection are unknown because not all infected pilgrims visited clinics and respiratory tract sampling was not performed on all clinic patients. The epidemiologic and virologic data gathered during WYD2008 highlight the complexity of an influenza outbreak within a large mass gathering. At least 6 distinct viruses circulated among pilgrims during WYD2008; 2 distinct influenza A (H1N1) viruses (oseltamivir-resistant and -sensitive), 2 distinct influenza A (H3N2) viruses (Australian/New Zealand and European clusters), and 2 distinct influenza B viruses (B/Malaysia–like and B/Florida–like). Different viruses were more likely to circulate in different groups within the pilgrim population, which suggests that exposure was not random but influenced by country of origin and travel before WYD2008.

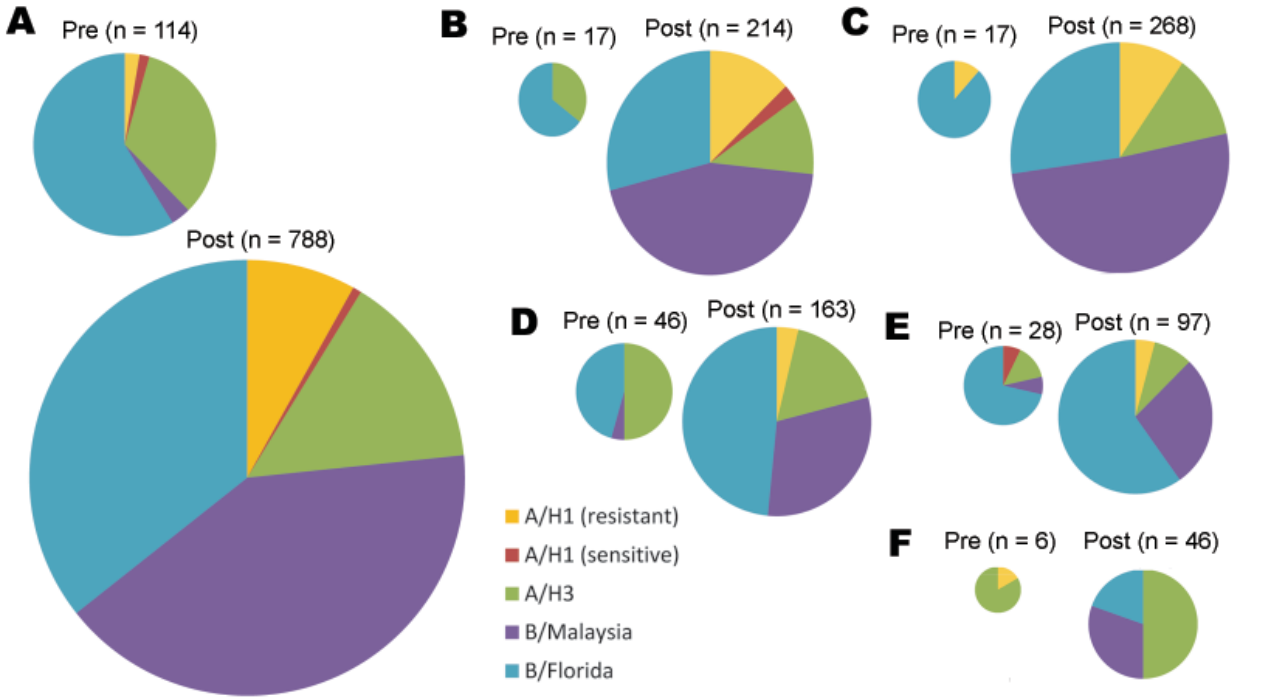

Figure 2. Relative effects of different influenza viruses before (pre) and after (post) World Youth Day 2008 for A) Australia; B) Western Australia; C) South Australia and Northern Territory; D) Queensland; E) New South Wales and Australian Capital Territory; and F) Victoria and Tasmania. The size of each pie chart is approximately proportional to the number of virus isolates analyzed from each region.

Mass gatherings with attendees traveling from overseas allow for the introduction of novel influenza viruses as well as the amplification of preexisting community strains. Among the strains identified during WYD2008, influenza A (H3N2) viruses (Australian/New Zealand cluster) and B/Florida-like viruses appear closely related to viruses that were circulating in the community before WYD2008. Although rarely isolated before WYD2008, oseltamivir-resistant influenza A/H1N1 and B/Malaysia–like viruses isolated from pilgrims are potentially related to viruses cultured from nonpilgrims in South Australia, Victoria, and Queensland, raising the possibility of local acquisition rather than introduction from overseas. Other viruses, such as influenza A (H3N2) (European cluster), were not detected in Australia before WYD2008 and thus appear to have been introduced with the pilgrims.

Exploring the influence of a mass gathering on community influenza activity is complex, especially when numerous viruses circulate during such an event. In 2008, the total number of laboratory-confirmed influenza cases in Australia was 1.9× the 5-year average yet not significantly different from the number of cases diagnosed in 2007 (*19*). WYD2008 coincided with the start of the normal influenza season in Australia, which is usually greatest between July and September (*21*,*22*). Thus, the rapid rise in influenza A and B activity in all states after WYD2008 may have occurred despite WYD2008. To add to the complexity are the differing and unpredictable consequences of each individual viral subtype/strain. This complexity is illustrated by oseltamivir-resistant A (H1N1) and the B/Malaysia-like influenza viruses, which were rarely detected before WYD2008 but responsible for 24%–34% and 13%–35% of infections in pilgrims tested. Pilgrim groups had substantial contact with nonpilgrims through travel in Australia before and after WYD2008. Despite this contact, post-WYD2008 dissemination of influenza A (H1N1) was modest compared with dissemination of influenza B/Malaysia-like viruses (A [H1N1], 8.1% of all post-WYD2008 isolates; B/Malaysia, 41.0% of all post-WYD2008 isolates).

A potential explanation for the substantial effect of influenza B/Malaysia-like viruses observed post-WYD2008 is reduced community immunity to influenza B. In 2007, influenza B was an infrequent pathogen (6.9% of total isolates typed); influenza A (H3N2) and A (H1N1) viruses were most frequently detected (58.7% and 34.4% of total isolates typed or subtyped) (*21*). In the 2003–2006 influenza seasons, influenza B was responsible for a low prevalence of disease compared with influenza A (6%–35% of total influenza isolates typed) (*22*–*25*). Influenza A (H1N1) A/Solomon Island/3/2006–like, A (H3N2) A/Brisbane/10/2007–like, and B/Florida/4/2006–like viruses were chosen to be included in the Southern Hemisphere winter 2008 influenza vaccine. Both the low rate of influenza B activity in preceding seasons and mismatch of B/Malaysia–like viruses with

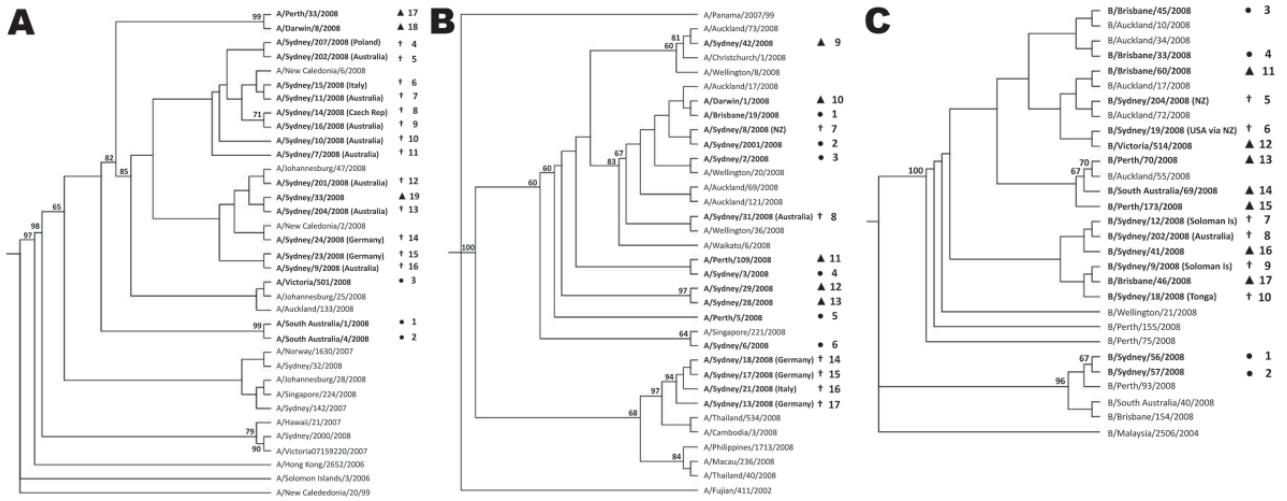

Figure 3. Phylogenetic trees illustrating relatedness of hemagglutinin sequences from influenza A (H1N1) (A), A (H3N2) (B), and B/Malaysia-like viruses (C) from pre–World Youth Day 2008 (WYD2008) Australian isolates (●), WYD2008 isolates (†), post-WYD2008 Australian isolates (▲), and related international isolates. Trees were constructed by using maximum-likelihood (DNAml) in PHYLIP. Only bootstrap values ≥60 are included.

the vaccine strain may have contributed to the greater B/Malaysia–like virus activity seen in community influenza in 2008.

Those who plan future mass gatherings need to consider the potential for influenza outbreaks. As observed, multiple viruses may circulate among those attending, thus increasing the opportunity for the emergence of novel reassortment viruses. Public health services need to be prepared to establish clinics rapidly and likely at many locations. Rapid diagnostic testing needs to be available, and laboratories need to be prepared for a rapid influx of specimens. Because viruses may be introduced, reliance on local rates of antiviral resistance may be misleading and resistance data from other countries may not be available. The circulation of oseltamivir-resistant seasonal influenza A (H1N1) and emergence of oseltamivir-sensitive influenza A pandemic (H1N1) 2009 virus (*26*) highlight the need for rapid typing of influenza viruses during outbreaks to guide the public health response. In influenza outbreaks in which circulating viruses include resistant seasonal influenza A (H1N1) virus, reliance on oseltamivir alone is likely to be insufficient. Influenza vaccination of all those attending should be recommended before mass gatherings, especially when held during the host countries' influenza season and given the likely emergence of antiviral resistance. Although pilgrims were encouraged to be vaccinated before attending WYD2008, the rate of recent vaccination was low. Because numerous viruses may circulate, vaccination may be insufficient to protect against all influenza strains.

This study has several limitations. First, clinics were established rapidly and involved numerous clinicians; thus, the prospectively collected data were not standardized. Second, only pilgrims who visited clinics with symptoms were assessed, and the proportion of the pilgrim population that had an influenza-like illness or asymptomatic infection is unknown. Third, the actual proportions of viruses detected and the pilgrim populations infected may differ from those described because respiratory tract sampling was restricted at several clinics after the identification of an outbreak. Fourth, Australian influenza type/subtype data used for comparison may be influenced by a referral bias from state-based reference laboratories. Finally, only representative isolates sent to the WHO Collaborating Centre underwent sequence analysis, so further related or unrelated isolates may not have been analyzed.

After epidemiologic and virologic assessment of the WYD2008 outbreak, we highlight the complexity of influenza outbreaks that occur during mass gatherings with numerous viruses co-circulating among attendees. Mass gatherings enable introduction of novel influenza strains into the local population and the amplification of circulating local seasonal influenza strains, or both, thereby increasing the opportunity for novel reassortment influenza viruses to emerge. This introduction or amplification of viruses in contained outbreaks may alter seasonal influenza activity subsequent to a mass gathering. The resultant effect on seasonal influenza activity is influenced by many competing forces, including population movement and preexisting immunity, and thus remains unpredictable. The potential for a substantial influenza outbreak needs to be considered before all mass gathering events, particularly when hosted during the months of peak influenza activity (*27*,*28*). Greater flexibility by public health and hospitals is required to appropriately manage and contain these outbreaks.

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Effects of Pneumococcal Conjugate Vaccine 2 Years after Its Introduction, the Netherlands

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In the Netherlands, the 7-valent pneumococcal conjugate vaccine (PCV-7) was implemented in a 3+1-dose schedule in the national immunization program for infants born after April 1, 2006. To assess the vaccine's effectiveness, we compared disease incidence before and after vaccine implementation (June 2004–June 2006 and June 2006–June 2008, respectively). We serotyped 2,552 invasive pneumococcal isolates from throughout the Netherlands, covering 25% of the country's population. Clinical characteristics were extracted from hospital records. After June 2006, vaccine-serotype invasive pneumococcal disease (IPD) decreased 90% (95% confidence interval [CI] 68%–97%) in children age eligible for PCV-7; simultaneously, however, non–vaccine-serotype IPD increased by 71% (not significant), resulting in a 44% total net IPD reduction (95% CI 7%–66%). IPD rates did not change for other age groups. In the Netherlands, PCV-7 offered high protection against vaccine-serotype IPD in vaccinated children, but increases of non–vaccine-serotype IPD reduced net vaccine benefits.

Streptococcus pneumoniae is a leading cause of invasive infections, such as meningitis, septicemia, and bacteremia, and of more common respiratory tract infections, such as pneumonia and otitis media. Young children and elderly persons are at particularly high risk for pneumococcal infection (*1*). In the United States, the introduction in 2000 of the CRM197-conjugated 7-valent pneumococcal vaccine (PCV-7) resulted in a 77% reduction in 2005 of invasive pneumococcal disease (IPD) in children <5 years of age from IPD rates reported in 1998–1999 (*2*). IPD rates in children decreased mostly within the first 2 years after introduction of PCV-7; leveled off in 2002; and then stabilized, despite an ongoing decrease of vaccine-serotype IPD, due to a gradual increase of non–vaccine-serotype IPD, particularly serotype 19A (*2*,*3*). In addition, use of the vaccine in children was associated with reduced IPD rates for unvaccinated age groups, which resulted from reduced nasopharyngeal colonization of vaccine-serotype *S. pneumoniae* in vaccinated children and concomitant reduced transmission (*4*,*5*). The cost effectiveness of herd immunity conferred by the conjugate vaccine in the United States prompted implementation of the vaccine in the Netherlands (*6*).

Data from the United States concerning both direct and indirect vaccine benefit, however, cannot be translated indiscriminately to European countries because of several major differences. Vaccine-serotype coverage by PCV-7 was lower in European countries (60%–70%) than in the United States (>80%) (*7*), which may leave more room for non–vaccine-serotype replacement in European countries. Second, in the Netherlands (as in most European countries), baseline IPD incidence rates are based mainly on culture-confirmed cases in hospitalized children, resulting in markedly lower IPD incidence rates for young children in the Netherlands than for those in the United States, where blood samples are cultured for more patients. Before introduction of PCV-7 in the Netherlands, overall IPD rates were 35 cases/100,000 children <2 years of age, of which 15 cases/100,000 children were meningitis (*1*). In contrast, in the United States, IPD incidence before introduction of

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PCV-7 peaked at 188 cases/100,000 children <2 years of age in 1998–1999 (*5*), and 10 cases/100,000 children in that age group were meningitis (*8*).

Consequently, introduction of PCV-7 may have affected IPD incidence in European countries differently than in the United States. (*9*). To assess the effectiveness of PCV-7 on IPD in the Netherlands, we evaluated the incidence and clinical syndromes of IPD in PCV-7–vaccinated and –unvaccinated children and in other age groups during the first 2 years after implementation of PCV-7.

Materials and Methods

Surveillance and Data Collection

PCV-7 was introduced into the Netherlands' national immunization program (NIP) in June 2006 and was recommended for all infants born after April 1, 2006, at 2, 3, 4, and 11 months of age (*10*). Our study comprised all patients with culture-confirmed IPD during June 1, 2004–June 1, 2006 (preimplementation period) and June 1, 2006–June 1, 2008 (postimplementation period). Isolates were serotyped by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM), which collects nationwide bacterial isolates from blood, cerebrospinal fluid (CSF), and/or other normally sterile bodily fluids for laboratory-based surveillance. Isolates from all patients with IPD were submitted by 9 sentinel laboratories throughout the country. These laboratories covered ≈4.074.412 and ≈4.090.233 residents in the preimplementation period and postimplementation period, respectively, representing ≈25% of the population of the Netherlands. Laboratories were selected on the basis of their reliability for submitting pneumococcal isolates; they submitted ≈90%–95% of the pneumococcal isolates from CSF and ≈83% of pneumococcal isolates from blood (*1*).

Isolates were serotyped as previously described, by using antiserum from the Statens Serum Institute (Copenhagen, Denmark) (*1*). Isolates with serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, the serotypes contained in PCV-7, were considered vaccine serotypes. All other serotypes were considered non–vaccine-serotypes.

Clinical Characteristics

Nearly all (97%–98%) IPD cases were in hospitalized patients. We retrospectively abstracted information about their clinical syndromes and underlying conditions from hospital records. Clinical syndromes were categorized as meningitis or nonmeningitis IPD (invasive pneumonia, IPD with other focus, and bacteremia without focus). Meningitis was defined as CSF culture positive for *S. pneumoniae* (or positive CSF by PCR) and/or clinical diagnosis of meningitis in combination with a blood culture positive for *S. pneumoniae*. Invasive pneumonia was physician-diagnosed pneumonia and a blood culture positive for *S. pneumoniae*. IPD with other focus was an *S. pneumoniae*–positive culture of blood or other normally sterile body fluid in combination with a clinical focus other than meningitis or pneumonia. For bacteremia without focus, no clinical focus was identified. Underlying conditions were classified as immunocompromised conditions or other comorbidities, as described previously (*1*). Case fatality was defined as in-hospital death and/or death within 30 days after the first reported blood/CSF culture positive for *S. pneumoniae*.

Statistical Analyses

To study the effectiveness of the vaccination program, we compared age-specific incidences during the preimplementation and postimplementation periods. Incidence rates of IPD were calculated as number of cases per 100,000 persons per year by using 25% of the Dutch population on January 1 for each considered year, accounting for the 25% coverage of surveillance data. Changes in incidence rates from the preimplementation to the postimplementation period were presented as incidence rate ratio with 95% confidence intervals (CI) and as percent changes. We compared the preimplementation and postimplementation periods with regard to distribution of clinical syndromes, comorbidities, and outcomes. Theoretical coverage of IPD was based on data from the preimplementation and postimplementation periods for future 10-valent PCV (PCV-10, covering PCV-7 serotypes plus serotypes 1, 5, and 7F) and 13-valent PCV (PCV-13, covering PCV-10 serotypes plus serotypes 3, 6A, and 19A). Proportions were tested with χ^2 or Fisher exact tests, as appropriate. We considered $p < 0.05$ to be significant. Statistical analyses were performed with SAS version 9.1.3 (SAS Institute, Cary, NC, USA), Excel 2007 (Microsoft, Redmond, WA, USA), and Episheet (*11*).

Results

During the study period, the NRLBM received 2,649 *S. pneumoniae* isolates: 1,297 during the preimplementation period and 1,352 during the postimplementation period. Medical records were assessed for 1,235 (95%) cases during the preimplementation period and for 1,317 (97%) cases during the postimplementation period. Pneumococcal serotype was available for 1,225 and 1,304 cases (both 99%), respectively.

IPD Incidence

Overall incidence of IPD remained stable; 15.9 vs. 15.0 cases/100,000 persons during the postimplementation and preimplementation periods, respectively. Incidence of vaccine-serotype IPD did not change significantly. For non–vaccine-serotype IPD, incidence increased 13% (95% CI 2%–26%; p = 0.02) (Table). In children <2 years of age,

Table. Incidence rates of invasive pneumococcal diseases before and after implementation of 7-valent pneumococcal conjugate vaccine, the Netherlands*

Serotypes by patient age group, y	Preimplementation period (June 2004–June 2006)		Postimplementation period (June 2006–June 2008)		Preimplementation vs. postimplementation	
	No. cases	Rate	No. cases	Rate	IRR (95% CI)	p value†
Total						
All ages	1,225	15.0	1,304	15.9	1.06 (0.98–1.15)	0.14
<2	68	34.5	42	22.5	**0.65 (0.44–0.96)**	**0.006**
2–4	25	8.1	26	8.7	1.07 (0.62–1.86)	
5–49	206	4.1	231	4.7	1.13 (0.94–1.37)	
50–64	254	16.7	292	18.5	1.11 (0.94–1.31)	
≥65	672	58.8	713	60.2	1.02 (0.92–1.14)	
Vaccine serotypes‡						
All ages	570	7.0	561	6.9	0.98 (0.87–1.10)	
<2	48	24.3	15	8.0	**0.33 (0.19–0.59)**	**<0.0001**
2–4	17	5.5	17	5.7	1.03 (0.53–2.02)	
5–49	69	1.4	70	1.4	1.02 (0.73–1.43)	
50–64	114	7.5	129	8.24	1.09 (0.85–1.40)	
≥65	322	28.2	330	27.9	0.99 (0.85–1.15)	
Nonvaccine serotypes§						
All ages	656	8.0	743	9.1	**1.13 (1.02–1.26)**	**0.02**
<2	20	10.1	27	14.5	1.43 (0.80–2.55)	
2–4	8	2.6	9	3.0	1.16 (0.45–3.01)	
5–49	137	2.8	161	3.3	1.19 (0.94–1.49)	
50–64	140	9.2	163	10.3	1.12 (0.89–1.41)	
≥65	350	30.6	383	32.4	1.06 (0.91–1.22)	

*Rate is cases/100,000 persons. IRR, incidence rate ratio; CI, confidence interval. **Boldface** indicates significant differences (p<0.05).
†p values shown <0.15; incidence rates pre vs. postimplementation period. Calculated by using Fisher exact test; all p values are 2 sided.
‡*Streptococcus pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F.
§All other *S. pneumoniae* serotypes.

including those not vaccinated or incompletely vaccinated, the incidence of IPD decreased 35% (95% CI 4%–56%; p = 0.006), from 34.5 cases/100,000 persons in the preimplementation period to 22.5 cases/100,000 persons in the postimplementation period (Table). Incidence of vaccine-serotype IPD declined by 67% (95% CI 41%–81%; p<0.0001), from 24.3 to 8.0 cases/100,000 persons. In contrast, non–vaccine-serotype IPD incidence increased, but not significantly, from 10.1 to 14.5 cases/100,000 persons (p = 0.40).

Among children born after April 1 2006 (i.e., age-eligible for vaccination according to the NIP), the incidence rate of vaccine-serotype IPD in the postvaccination period (2.4 cases/100,000 persons) decreased 90% (p<0.0001) compared with that for an age-matched group in the preimplementation period (24.2 cases/100,000) (Figure 1). Although not significant because of low numbers, the incidence of non–vaccine-serotype IPD had risen by 71%, from 9.8 to 16.8 cases/100,000 persons (p = 0.12), leading to a total net reduction of 44% (95% CI 7%–66%; p = 0.02) in the birth group age-eligible for vaccination.

Three vaccine-serotype IPD cases occurred among children born after April 1, 2006; 2 cases after 1 vaccine dose (serotypes 9V and 23F) and 1 case within 1 week after the second dose (serotype 9V, isolated from CSF). In infants <2 years of age born before April 1, 2006 (i.e., age-ineligible for PCV-7), no changes occurred in vaccine- or non–vaccine-serotype IPD rates in the postimplementation period compared with those for age-matched children in the preimplementation period.

Serotype Distribution

After introduction of PCV-7, for all vaccine serotypes, the number of IPD cases among the total population remained stable, except for serotype 19F (44 vs. 23 cases; p = 0.004). Also, proportions of non–vaccine-serotype 1 and 22F significantly increased (Figure 2). Among children born after April 1, 2006, serotypes 1 and 7F increased in comparison with those for age-matched infants in the preimplementation period (Figure 3). For serotype 6A, 6C and 19A, no significant changes occurred in any age group.

Clinical Characteristics

Among children <2 years of age, incidence rates decreased for all clinical syndromes to approximately the same extent (Figure 4). Rates of meningitis declined 34% from 14.7 to 9.6 cases/100,000 children (29 vs. 18 cases) and of nonmeningitis IPD 35% from 19.8 to 12.9 cases/100,000 children in this age group (39 vs. 24 cases). Of these children, 30% (20/66) had comorbidities in the preimplementation period, compared with 9% (4/44) in the postimplementation period (p<0.001). In all other age

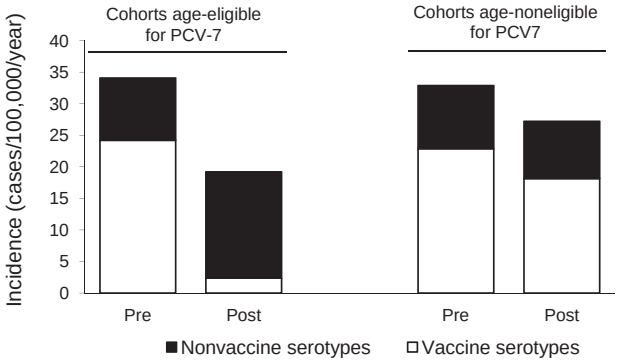

Figure 1. Incidence of invasive pneumococcal disease in children <2 years of age in the birth group born after April 1, 2006 (age eligible for 7-valent pneumococcal conjugate vaccine [PCV-7]) and children born before April 1, 2006 (age noneligible for PCV-7), in the postimplementation period compared with age-matched children in the preimplementation period, the Netherlands. Incidence per 100,000 children <2 years of age per year; Pre, preimplementation period (June 2004–June 2006); post, postimplementation period (June 2006–June 2008).

groups, clinical syndromes did not change from the preimplementation to the postimplementation period, except for a 124% rise in rates of non–vaccine-serotype meningitis for persons 5–49 years of age (95% CI 19%–320%; p = 0.01). The proportions of adult patients with comorbidities and immunocompromising conditions were similar in the preimplementation and postimplementation periods: 71% vs. 74% and 19 vs. 22%, respectively. For the vaccinated group of children, the case-fatality rate remained stable (9.3% vs. 8.3%) in the preimplementation and postimplementation periods, respectively. In other age groups, case-fatality rates did not differ (data not shown).

Estimated Coverage by Future Vaccines

Among vaccination-eligible children, the additional coverage rates in the preimplementation and postimplementation periods were 2.2% (1/45 cases) and 54.2% (13/24 cases) for PCV-10 (p<0.0001). For PCV-13, they were 19.6% (8/45 cases) and 66.7% (16/24 cases) (p<0.001) (Figure 5).

Discussion

The total net IPD reduction of 35% among children <2 years of age observed in the Netherlands differs from the favorable results reported in the United States, where a 69% decrease in IPD within the first 2 years after PCV-7 introduction was reported despite lower vaccine uptake (i.e., estimates of national immunization coverage) rates in the United States than in the Netherlands (*2*,*12*). US estimates for PCV-7 uptake among children born in 2001 who received ≥1 and ≥3 doses were 89% and 68%, respectively (2). In contrast, in the Netherlands, 94.4% of all infants born in 2006 were fully vaccinated at 2 years of age (*12*). Also, the decrease in meningitis incidence in the Netherlands was lower than that for US infants <2 years of age (34% vs. 59%), whereas during the preimplementation period, meningitis incidence was comparable in the 2 countries. In the first 2 years after implementation in the United States, IPD requiring hospitalization decreased 63% in children <2 years (*5*). In contrast, all IPD in this age group decreased 35%, and almost all reported IPD cases occurred in hospitalized patients.

The difference between the impact of PCV-7 in the Netherlands and the United States may be attributable to the lower proportion of vaccine-serotype cases covered by PCV-7 before implementation in the Netherlands and in Europe. Reports about PCV-7 effectiveness by other European countries support this observation. In Germany and Norway, PCV-7 effectively prevented disease in the youngest age groups during the first years after implementation, without major increase of non–vaccine-serotype IPD (*13*,*14*). In Spain, vaccine-serotype IPD decreased after PCV-7 implementation; however, non–vaccine-serotype IPD increased (*15*,*16*). In France, 3 years after PCV-7 introduction, overall IPD cases decreased 21% among children of ≤2 years of age, when 44%–56% of children were vaccinated (*17*). In addition, similar to findings in the Netherlands, a simultaneous increase in IPD from non–vaccine-serotype pneumococci reduced the net benefit of vaccination. Differences in surveillance systems, temporal fluctuations of circulating serotypes, antimicrobial drug resistance and penicillin susceptibility of circulating

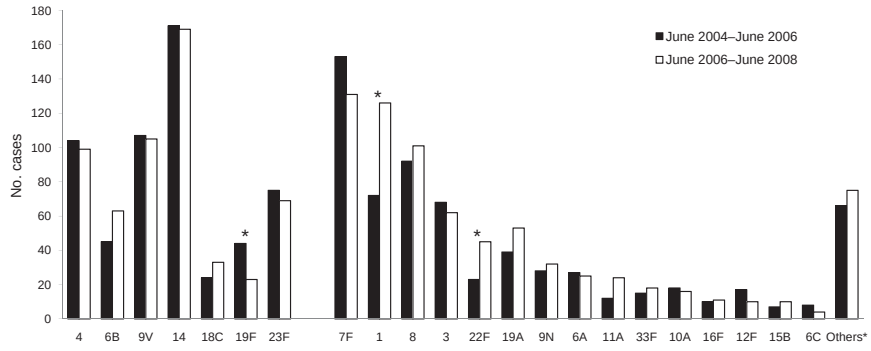

Figure 2. Serotype distribution of invasive pneumococcal disease with regard to preimplementation and postimplementation of 7-valent pneumococcal conjugate vaccine (PCV-7); among persons of all ages, the Netherlands. Preimplementation period June 2004–June 2006; postimplementation period June 2006–June 2008; *p<0.05; proportion of serotypes preimplementation vs. postimplementation period. Calculated using Fisher exact test; all p values are 2 sided.

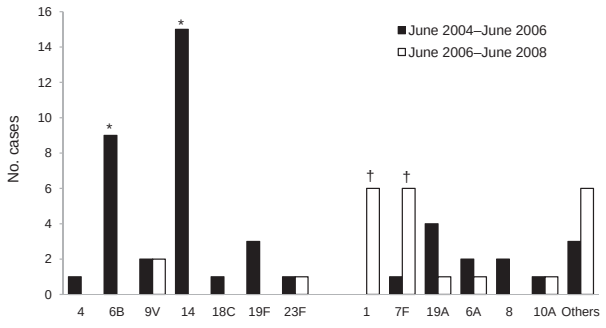

Figure 3. Serotype distribution of invasive pneumococcal disease cases among children born after April 1, 2006 (age eligible for 7-valent pneumococcal conjugate vaccine [PCV-7]) in the postimplementation period compared with age-matched children in the preimplementation period, the Netherlands. Preimplementation period, June 2004–June 2006; postimplementation period, June 2006–June 2008; other serotypes are 15A, 16F, 22F, 3, 33F, 5, and 9N. *p<0.05; preimplementation vs. postimplementation periods. Proportions calculated using Fisher exact test; all p values are 2 sided.

pneumococcal strains, vaccination schedules, vaccine uptake, and blood sampling practices also may play a role in the differences between countries (*9*,*18*). Like Norway, a reduced-dose PCV-7 schedule has been introduced in the United Kingdom. In the first years after implementation, surveillance data from the United Kingdom have tended to show a major decline in vaccine-serotype IPD in infants <2 years of age concomitant with a substantial rise in non–vaccine-serotype IPD, reducing net vaccine benefits (*19*,*20*). Unlike in the United States, where IPD in children <2 years of age stabilized within 2 years after introduction of the vaccine (*2*), in Europe and in our study, incidence has not yet stabilized. Our results emphasize the need for continued surveillance to monitor the long-term public health benefits of the vaccination program.

Despite the high vaccination uptake in the Netherlands, we observed no indication of herd immunity in other age groups during the first 2 years after implementation, except for a decrease in serotype 19F. This observation may be explained by the relatively short evaluation period of 2 years, a relatively small vaccinated group (2.25%) of the total population, and lack of a catch-up program for older children. In Australia and the United Kingdom, which have catch-up programs for children ≤2 years of age, decreases in vaccine-serotype IPD in unvaccinated children within 3 years after PCV-7 implementation have been reported (*21*,*22*).

The small increase we found in non–vaccine-serotypes 1 and 7F among vaccinated children could be attributed to temporal fluctuations (*18*,*23*). The numbers in our study were too small and the period we studied too short to enable us to draw firm conclusions about changes in serotype-specific incidence. Serotype 1 also has increased in other age groups and may cause local outbreaks, as observed in other countries before the implementation of PCV-7 (*24*,*25*). In our study, we could not find evidence of outbreaks associated with serotype 1.

The increase in non–vaccine-serotype IPD in the vaccinated age group was not explained by more children with comorbidities or immunocompromised conditions in the years after introduction and not associated with a change in the case-fatality rate. Longer follow-up is needed to assess whether this increase and that in non–vaccine-serotype IPD in the overall population are temporary or are vaccine related. Several countries have suggested that use of PCV-7 might enhance the emergence of serotype 19A pneumococcal clones, often associated with penicillin resistance (*26*). In the Netherlands, where use of antimicrobial drugs is restricted, few penicillin-resistant pneumococcal isolates were received during the study period; 98.8% were susceptible to penicillin (MIC ≤0.06 mg/L), 0.8% were intermediately susceptible (0.06–1.0 mg/L), and 0.4% were resistant (>1.0 mg/L). We did not see a prominent increase in serotype 19A among patients; only 1 isolate was penicillin resistant (>1.0 mg/L) in the first 2 years after PCV-7 implementation. However, increase in serotype 19A pneumococci was found in a randomized controlled study of nasopharyngeal

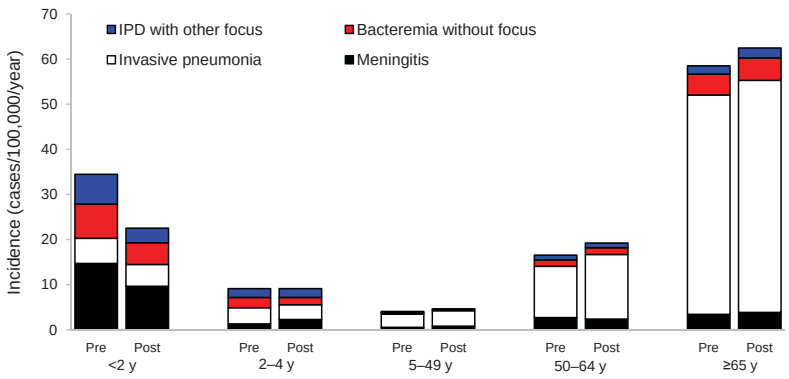

Figure 4. Age group–specific distribution of clinical invasive pneumococcal disease (IPD) syndromes in the preimplementation and postimplementation periods of 7-valent pneumococcal conjugate vaccine (PCV-7), the Netherlands. Incidence is IPD cases per 100,000 persons per year. Pre, preimplementation period (June 2004–June 2006); post, postimplementation period (June 2006–June 2008).

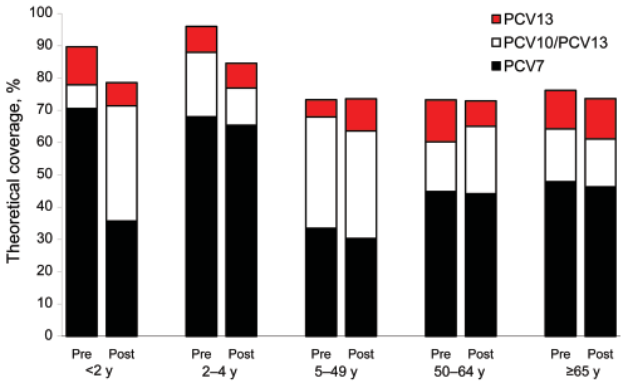

Figure 5. Age group–specific theoretical coverage of pneumococcal conjugate vaccines during the preimplementation and postimplementation periods of 7-valent pneumococcal conjugate vaccine (PCV-7), the Netherlands. IPD, invasive pneumococcal disease; PCV-10/PCV13, additional coverage by PCV-10 and PCV-13; PCV-13, additional coverage by PCV-13 alone; pre, preimplementation period (June 2004–June 2006); post, postimplementation period (June 2006–June 2008).

carriage among vaccinated children compared with unvaccinated controls before national implementation of PVC-7 (*27*). Also, no changes in distribution of serotype 6A or 6C were reported. Theoretical coverage of the future conjugate vaccines PCV-10 and PCV-13 increased in the postimplementation period in vaccination-eligible children. In all other age groups, no changes were observed. Future vaccines need to be considered to improve the net benefit of immunization against pneumococcal diseases.

Some limitations should be acknowledged. Although our study covered ≈25% of the population of the Netherlands, numbers of IPD cases and serotype distribution are small and need cautious interpretation. After 2 years, final vaccine benefits cannot be established. Furthermore, distribution of serotypes among invasive pneumococci may fluctuate over time, and temporal trends in serotype may vary across geographic regions and independent of PCV-7 implementation (*18*,*23*). Although the 9 sentinel laboratories submitted 25% of all pneumococcal isolates received by the NRLBM (nationwide coverage 95%), the population under surveillance might be overestimated because the laboratories were selected on reliability of stable pneumococcal isolates submission over the years. Second, our surveillance system depended on how well the 9 sentinel laboratories were submitting their isolates, and small shifts in the proportion of submission to the NRLBM cannot be excluded (*14*). Blood culture rates may have influenced IPD incidence reported in this study (*28*). However, these changes are not likely to be substantial. National IPD incidence rates estimated from the number of isolates submitted by the 9 sentinel laboratories are similar to those of neighboring countries, e.g., Denmark and the United Kingdom, that have comparable health system practices (*29*). The rates of submission of blood and CSF isolates for children <5 years of age have been stable in the Netherlands for the past 10 years at ≈90%. Enhanced surveillance with such high submission rates and stable overall IPD rates cannot explain the 71% increase of nonvaccine serotypes. Also, and most important, the changes in IPD serotype distribution occurred only in vaccination-eligible infants. No changes in serotype distribution or signs of herd immunity were observed in unvaccinated infants. A potential bias by enhanced awareness would cause differences also in this group. Long-term surveillance data will elucidate whether the changes in serotype distribution in vaccinated and unvaccinated persons remain. Lastly, in the Netherlands, no changes in diagnostic methods or blood culture practices have been implemented recently.

Strengths of our study include the detailed information about the IPD cases and the established high degree of vaccine uptake for the NIP in the Netherlands (≈95% of infants of ≤1 year of age are fully vaccinated) (*12*). Vaccination with the 23-valent pneumococcal polysaccharide vaccine has not been routinely recommended for elderly persons, and uptake has been negligible in the Netherlands; thus, any influence of this vaccine can be excluded (*30*). The low proportion of penicillin-resistant pneumococcal isolates and the densely living but relatively homogeneous population make the Netherlands particularly suitable for describing vaccine effects. Our study provides accurate data from a representative group of the Dutch population with fairly detailed information about the distribution of clinical syndromes and presence of comorbidities.

Shortly after introduction of PCV-7 vaccination for infants, the direct vaccine effectiveness on IPD caused by vaccine-serotype pneumococci appeared high in the Netherlands. However, the net benefit is partly offset by the increased incidence of nonvaccine serotypes. For this reason, future conjugate vaccines may be valuable in further reducing IPD incidence. These results further emphasize the need for ongoing surveillance.

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Dr Rodenburg works at the Wilhelmina Children's Hospital/University Medical Center in Utrecht, the Netherlands. This work was part of a PhD research project. His research interests include effectiveness of pneumococcal conjugate vaccinations and the development of immunologic protection against pneumococcal disease.

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Rapid Influenza Antigen Test for Diagnosis of Pandemic (H1N1) 2009

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We compared the QuickVue Influenza test with PCR for diagnosing pandemic (H1N1) 2009 in 404 persons with influenza-like illness. Overall sensitivity, specificity, and positive and negative predictive values were 66%, 84%, 84%, and 64%, respectively. Rapid test results should be interpreted cautiously when pandemic (H1N1) 2009 virus is suspected.

Since its emergence, the pandemic (H1N1) 2009 virus has spread rapidly throughout the world. To diagnose influenza at the point of care, many clinicians rely on commercial rapid enzyme immunoassay tests, which are currently unable to differentiate between influenza A virus subtypes (*1*). Compared with PCR and viral culture, the sensitivity of rapid tests for seasonal influenza varies from 70% to 90% in children and <40% to 60% in adults (*2*,*3*). The positive and negative predictive values (PPVs and NPVs) of rapid tests depend on the prevalence of influenza viruses among the population being tested (*2*,*3*).

We compare PCR with a rapid influenza test to better characterize the diagnostic utility of the rapid test during the current pandemic. The QuickVue Influenza test (Quidel Corp., San Diego, CA, USA) detects influenza A and B viruses but does not distinguish between them. Clinicians may use the test in their offices because it is waived from Clinical Laboratory Improvements Amendment requirements based on documentation that test results by persons without formal laboratory training are in concordance with results by trained laboratorians.

The Study

The California Department of Public Health (CDPH) supplied QuickVue Influenza test kits to clinicians participating in the Centers for Disease Control and Prevention (CDC) Sentinel Provider Influenza Surveillance Program. Sentinel providers performed the QuickVue Influenza test on a first respiratory specimen obtained from outpatients with influenza-like illness (fever ≥100°F and cough and/or sore throat) using the foam swab provided by QuickVue. Clinicians collected a second respiratory specimen using a sterile Dacron swab that was stored in viral transport media at 4°C for ≤72 hours before shipment to CDPH. Sentinel providers recorded information about patient demographics, symptoms, and QuickVue test results on a standardized specimen collection form.

At CDPH, specimens were tested by an influenza A universal real-time reverse transcription–PCR (rRT-PCR) assay with an analytical sensitivity (50% tissue culture infective dose /PCR input) of 0.51 for influenza A (*4*). If influenza A virus nucleic acid was detected, subtyping for human influenza A (H1 and H3) was performed. Specimens negative for any subtype were tested for pandemic (H1N1) 2009 by using a rRT-PCR detection panel provided by CDC. For all PCR testing, a cycle threshold (Ct, the cycle count at which amplified product yielded a detectable fluorescent signal) ≤40 was interpreted as positive. Sensitivity, specificity, predictive values, likelihood ratios, and posttest probabilities were estimated according to standard definitions (*5*). This activity was reviewed by the California Committee for the Protection of Human Subjects and determined to be a public health response that did not require institutional review board approval.

From May 4 to November 19, 2009, a total of 703 specimens were collected, including swabs from nares (293), nasopharynx (178), oropharynx (3), a mixture of sites (227), and unspecified sites (2). During this same period, statewide surveillance detected pandemic (H1N1) 2009 in 30%–50% of patients with influenza-like illnesses tested and 92%–100% of influenza viruses identified.

The median age of patients with influenza-like illness was 19 years (range 0–80 years). The median time from illness onset to specimen collection was 2 days (range 0–20 days). Of 703 specimens tested, 417 came from patients who had positive PCR results for influenza; 13 had seasonal influenza A subtypes, including 9 A/H1 and 4 A/H3; and 404 patients had pandemic (H1N1) 2009. Of these 404 patients, 266 (66%) had positive results and 138 (34%) had negative results by rapid antigen test (Table). Of 299 patients in which pandemic (H1N1) 2009 was not detected by PCR, 49 (16%) were positive and 250 (84%) were negative by the rapid antigen test. The prevalence of pandemic (H1N1) 2009 infection in all samples was 57%. The overall sensitivity, specificity, PPV, and NPV of the QuickVue Influenza Rapid Test for 2009 (H1N1) influenza when compared with PCR, regardless of the timing of collection, were 66%, 84%, 84%, and 64%, respectively, with a positive test result increasing the posttest probability from

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Table. Performance of rapid antigen test compared with PCR in the diagnosis of pandemic (H1N1) 2009*

Parameter	All specimens	Patient age <18 y†	Patient age ≥18 y†
No. rapid test positive, PCR positive	266	131	130
No. rapid test positive, PCR negative	49	19	28
No. rapid test negative, PCR positive	138	62	74
No. rapid test negative, PCR negative	250	78	166
Total no. tested	703	290	398
Prevalence of PCR positives in sample	0.57	0.67	0.51
Sensitivity	0.66	0.68	0.64
Specificity	0.84	0.80	0.86
Positive predictive value	0.84	0.87	0.82
Negative predictive value	0.64	0.56	0.69
Positive likelihood ratio (95% CI)	4.0 (3.1–5.2)	3.5 (2.3–5.3)	4.4 (3.1–6.3)
Posterior probability of positive test result (95% CI)	0.84 (0.81–0.88)	0.87 (0.82–0.91)	0.82 (0.76–0.87)
Negative likelihood ratio (95% CI)	0.41 (0.35–0.47)	0.40 (0.32–0.50)	0.42 (0.35–0.51)
Posterior probability of negative test result (95% CI)	0.36 (0.32–0.39)	0.44 (0.39–0.50)	0.31 (0.27–0.35)

*CI, confidence interval.
†Does not include results for 15 case-patients where age was not recorded.

57% to 84% and a negative test result decreasing it to 36%. The sensitivity, specificity, PPV, and NPV of the rapid test compared to PCR for persons <18 years of age were 68%, 80%, 87%, and 56%, respectively, and for persons ≥18 years were 64%, 86%, 82%, and 69%, respectively.

Ct values were available for 389 specimens in which pandemic (H1N1) 2009 virus was detected by PCR; of these, the median influenza A PCR Ct value was 26 for 135 specimens with a negative rapid test result and 21 for 254 specimens with a positive rapid test result ($p<0.0001$); samples with higher viral loads were more likely to be positive by rapid test (Figure). Even so, ≈25% of PCR-positive, rapid test–negative specimens had Ct values <23.

Other smaller studies have found comparable sensitivities, but higher specificities, for rapid antigen tests for pandemic (H1N1) 2009. In a CDC study of 45 samples provided by state laboratories, the sensitivity of all rapid tests was 40%–69%, including 69% for QuickVue Influenza A+B (*6*). Others have found the QuickVue rapid tests to have sensitivities of 51%–63% and specificities of 99%–100% (*7*–*9*). During a large cluster of school outbreaks in New York, NY, USA, the sensitivity and specificity of the Binax NOW (Inverness Medical International, Bedford, UK) rapid test were 17.8% and 93.6%, respectively (*10*). As we found, positive rapid antigen test results in other studies also appear to correlate with higher concentrations of pandemic (H1N1) 2009 virus (*6*,*11*,*12*).

Conclusions

Our findings illustrate the challenges clinicians face during the current pandemic. Because clinical symptoms of pandemic (H1N1) 2009 are nonspecific, definitive diagnosis requires confirmatory PCR testing, which, when available, often requires several days between specimen collection and reporting of results. Rapid antigen tests are the only current option for screening and diagnosis at the point of care. Current CDC guidelines recommend that high-risk and hospitalized infected patients be treated promptly with antiviral drugs and managed by using specific infection control precautions (*13*). Given the frequency of error found in this study, pandemic (H1N1) 2009 cannot be excluded solely because of a negative rapid antigen test result. Likewise, false-positive results, which would be expected to increase when the prevalence of influenza as a cause of influenza-like illness decreases, may result in unwarranted treatment and infection control measures that can be labor and resource intensive. Although rapid antigen tests are reported to have high specificity for seasonal influenza, our findings conflict with previous assumptions that rapid antigen tests are sufficiently specific to guide decisions about withholding antiviral treatment or chemoprophylaxis for pandemic (H1N1) 2009 (*2*).

A difference in swab types between rapid and PCR testing might have affected sensitivity of the rapid test re-

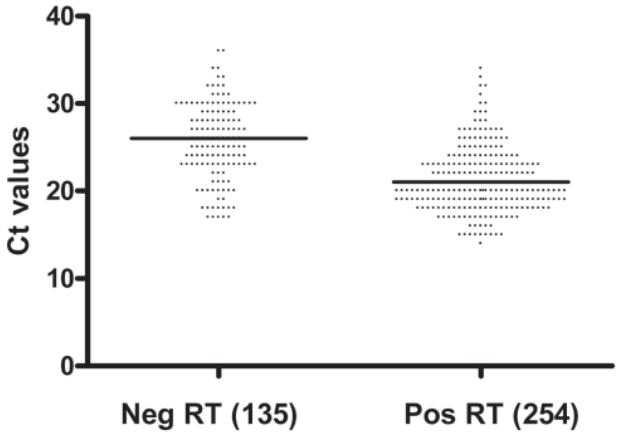

Figure. Comparison of cycle threshold (Ct) values for pandemic (H1N1) 2009 real-time reverse transcription–PCR-positive specimens (n = 389) with negative (neg) and positive (pos) rapid antigen test (RT) results. Solid lines represent median value for Ct.

sults. Likewise, although influenza B virus was detected in only 9 (0.09%) of 10,367 specimens during the 7.5 months of statewide surveillance, some rapid test results may have been interpreted as falsely positive due to infection with influenza B.

In conclusion, we found the QuickVue influenza test had suboptimal sensitivity and specificity for the detection of pandemic (H1N1) 2009 during a period of increased prevalence in California. This finding suggests that rapid test results that may lead to changes in clinical management or public health intervention should be confirmed with PCR. A strength of our study is its reflection of typical testing practices in outpatient settings and the need for reconsideration of the clinical application of rapid test results. The development of more accurate point-of-care tests for seasonal and pandemic (H1N1) 2009 infection is urgently needed.

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Multihospital Outbreak of *Clostridium difficile* Infection, Cleveland, Ohio, USA

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To determine whether a multihospital *Clostridium difficile* outbreak was associated with epidemic strains and whether use of particular fluoroquinolones was associated with increased infection rates, we cultured feces from *C. difficile*–infected patients. Use of fluoroquionolones with enhanced antianaerobic activity was not associated with increased infection rates.

Recent outbreaks of *Clostridium difficile* infection have been attributed to the emergence of an epidemic strain characterized as North American pulsed-field gel electrophoresis type 1 (NAP1) or restriction endonuclease assay group BI (*1*,*2*). Fluoroquinolone resistance is a hallmark of epidemic *C. difficile* isolates (*1*), and fluoroquinolone use has been associated with *C. difficile* infection (*2*–*9*). Because the C-8 methoxy fluoroquinolones gatifloxacin and moxifloxacin have enhanced antianaerobic activity, they might promote *C. difficile* infection to a greater degree than ciprofloxacin and levofloxacin (*10*). In 3 studies, substitution of gatifloxacin or moxifloxacin for levofloxacin was associated with an increase in *C. difficile* infection (*6*,*8*,*9*); in 2 of the 3 studies, a formulary change back to levofloxacin was associated with reduced *C. difficile* infection (*6*,*9*). However, ciprofloxacin and levofloxacin also have been associated with *C. difficile* infection (*2*–*5*,*7*).

Beginning in 2002, outbreaks of *C. difficile* infection occurred in several hospitals in the Cleveland, Ohio, USA, area. In response, the Ohio Department of Health (ODH) made *C. difficile* infection a reportable disease in 2006. One objective of the current study was to examine the magnitude of the outbreaks in Cuyahoga County, which comprises Cleveland and the surrounding area, and to determine whether the outbreaks were associated with epidemic BI/NAP1strains. A second objective was to examine whether use of gatifloxacin and/or moxifloxacin was associated with increased rates of *C. difficile* infection in healthcare facilities and to assess whether outbreaks correlated with formulary changes in fluoroquinolones.

The Study

We used the ODH website (www.odh.state.oh.us) to obtain rates (cases/10,000 patient-days) of initial *C. difficile* infections during January–December 2006 for the 22 hospitals in Cuyahoga County. All healthcare facilities in Ohio were required to submit *C. difficile* infection rates by using a standardized method of reporting. An initial case was defined as a first positive laboratory diagnostic test for *C. difficile*, pseudomembranes on endoscopy, or confirmatory histologic features from surgical or autopsy specimen. An infection that occurred >6 months after a previous infection was classified as an initial infection.

For a subset of 5 hospitals, up to 20 consecutive stool samples from individual patients with *C. difficile* infection were cultured for *C. difficile* (*11*). *C. difficile* isolates were tested for in vitro cytotoxin production and moxifloxacin susceptibility and analyzed for binary toxin gene *cdtB* and partial deletions of the *tcdC* gene (*11*–*13*). Molecular typing was performed by using PCR ribotyping (*11*). The 5 hospitals were 1 community hospital, 3 tertiary care facilities, and 1 Veterans Affairs hospital. Three of the hospitals had experienced large outbreaks of *C. difficile* infection in 2002–2003 (i.e., their *C. difficile* incidence doubled and their peak incidence was >20 cases per 1,000 discharges); the other 2 reported an increase in the proportion of cases that were fulminant. The infection control departments of each institution provided information about *C. difficile* infection rates, fluoroquinolones on formulary, and infection control measures for *C. difficile* during January 2000–December 2006.

Rates of *C. difficile* infection for 2006 were compared among hospitals with moxifloxacin or gatifloxacin versus those with levofloxacin on formulary as primarily fluoroquinolones used to treat respiratory infections. In addition, for 2 hospitals in the molecular typing analysis that had a formulary change from 1 respiratory fluoroquinolone to another, we used Poisson analysis to compare rates of *C. difficile* infection during the 12 months before and after the formulary change, with a lag of 1 month after the change. We analyzed data using SPSS statistical software version 10.0 (SPSS Inc., Chicago, IL, USA) and STATA 9.1 (StataCorp, College Station, TX, USA).

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Table. *Clostridium difficile* infection rates and healthcare facility characteristics according to respiratory fluoroquinolone on formulary, Cleveland, Ohio, USA, 2006*

Characteristic	Levofloxacin	Moxifloxacin	Neither†	Total
No. hospitals	13	8	1	22
No. beds, median (IQR)	232 (108–371)	361 (217–565)	1,008	316 (125–424)
Type of facility				
Tertiary care	0	2	1	3
Acute care	9	6	0	15
Long-term acute care	4	0	0	4
Hospital system				
System 1	1	5	0	6
System 2	11	0	1	12
Neither	1	3	0	4
No. cases of *C. difficile* infection	494	569	206	1,269
Patient-days	580,893	666,719	293,833	1,541,445
Rate of *C. difficile* infection/10,000 patient-days, median (IQR)	8.5 (7.8–9.3)	8.5 (7.8–9.2)	7.0 (6.1–8.0)	8.2 (7.8–8.7)

*IQR, interquartile range.
†Ciprofloxacin on formulary but no respiratory fluoroquinolone on formulary.

For the 18 adult acute-care hospitals and 4 long-term acute-care (LTAC) facilities in Cuyahoga County, the median *C. difficile* infection rate in 2006 was 7.3 (range 4.2–63.1 cases/10,000 patient-days). The highest rates were observed in 2 LTAC facilities. Six facilities (3 acute-care hospitals and 3 LTACs) had higher *C. difficile* infection rates than did each of the 5 hospitals in the molecular typing analysis.

A total of 64 toxigenic *C. difficile* isolates were cultured from feces samples obtained from 5 hospitals. Features of 42 (66%) isolates were consistent with epidemic BI/NAP1 strains (range 55%–83% for each facility), including amplification of the binary toxin gene *cdtB* and partial deletions in *tcdC* and resistance to moxifloxacin (MICs >32 μg/mL). By PCR ribotyping, we observed a characteristic banding pattern for isolates with features of the epidemic strain; 6 isolates with this banding pattern were confirmed as BI strains in the laboratory of D.G.

Of the 22 facilities, 8 used moxifloxacin as the primary respiratory fluoroquinolone, 13 used levofloxacin, and 1 did not have a respiratory fluoroquinolone on formulary. The *C. difficile* infection rate did not differ between facilities with levofloxacin (8.5 cases/10,000 patient-days, 95% confidence interval [CI] 7.8–9.3) and moxifloxacin (8.5 cases/10,000 patient-days, 95% CI 7.8–9.2) on formulary (p = 1) (Table). The facility that did not have a respiratory fluoroquinolone on formulary had a lower rate of *C. difficile* infection than the median rates for facilities that used levofloxacin or moxifloxacin. However, 8 facilities had lower *C. difficile* infection rates than did this institution.

Two of the 5 hospitals in the molecular typing analysis changed their formulary fluoroquinolones during the study period (Figure). Both hospitals made formulary changes from levofloxacin to gatifloxacin; however, the increase in *C. difficile* infection rates preceded the formulary change in each hospital. *C. difficile* infection rates did not differ significantly in the 12 months before and after the change from levofloxacin to gatifloxacin (relative risk [RR] 1.0, 95% CI 0.97–0.86; p = 0.973). For hospital 2 (Figure, panel B), a subsequent formulary change from gatifloxacin to levofloxacin was associated with a reduction in *C. difficile* infection (RR 0.59, 95% CI 0.51–0.70; p<0.001); an intervention to improve environmental cleaning with a 10% bleach solution occurred at the time of the formulary change.

Conclusions

Our findings provide further evidence that emergence of epidemic NAP1/BI strains in a geographic region may be associated with large multihospital outbreaks of *C. difficile* infection. Before the ODH decision to require mandatory reporting, many area hospitals were either not collecting surveillance data about *C. difficile* infection or were reluctant to share their rates. Therefore, we believe that mandatory public reporting of *C. difficile* infection rates provided a valuable tool to examine the full magnitude of the outbreaks and an incentive for hospitals with high rates to increase efforts to control infection. One area hospital recently reported that the ODH database underestimated the incidence of *C. difficile* infection (*14*), but this observation does not affect our conclusions because all facilities used the same surveillance definitions. Our findings do not support the hypothesis that use of moxifloxacin or gatifloxacin is associated with higher rates of *C. difficile* infection than is use of levofloxacin or ciprofloxacin.

Our analysis of formulary fluoroquinolones and *C. difficile* infection has several limitations. First, data on the amount of the fluoroquinolones used in the hospitals were not available. Second, analysis of hospital formularies does not account for the effects of fluoroquinolones used in long-term care facilities and among outpatients. Third, we did not assess confounding factors, such as use of other classes

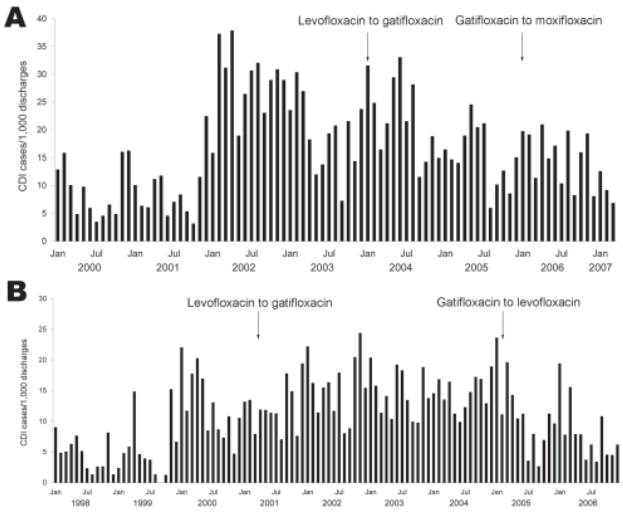

Figure. Rates of *Clostridium difficile* infection for hospital 1 (A) and hospital 2 (B). Arrows indicate the timing of the formulary changes in fluoroquinolone antimicrobial drugs.

of antimicrobial drugs and differing patient populations. Finally, studies that evaluate group-level effects may not reflect the biological effects at the individual-patient level. Additional studies are needed to evaluate the risk for *C. difficile* infection associated with different fluoroquinolones.

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Rickettsiae in Gulf Coast Ticks, Arkansas, USA

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To determine the cause of spotted fever cases in the southern United States, we screened Gulf Coast ticks (*Amblyomma maculatum*) collected in Arkansas for rickettsiae. Of the screened ticks, 30% had PCR amplicons consistent with *Rickettsia parkeri* or *Candidatus* Rickettsia amblyommii.

The Centers for Disease Control and Prevention identified Arkansas as a leading state for the incidence of Rocky Mountain spotted fever (causative agent *Rickettsia rickettsii*) and reported >15 cases per 1,000,000 persons in 2002 (*1*). Given the known cross-reactivity of serologic testing results for spotted fever group (SFG) rickettsia, it is unclear if cases outside the natural range of the vectors for *R. rickettsii* are misdiagnosed, if the pathogen is less virulent than previously suggested, or if additional rickettsiae are responsible for pathogenesis (*2*).

Recently, the Gulf Coast tick (*Amblyomma maculatum*) was identified as the primary vector of *R. parkeri*, a newly described pathogen that causes disease symptoms similar to Rocky Mountain spotted fever (*3*). *R. parkeri* has previously been identified in *A. maculatum* tick specimens collected in the southeastern United States (*4*) and from a human biopsy specimen in Virginia, USA (*5*). We have identified *A. maculatum* ticks collected from canids, felids, white-tailed deer, and a cow from locations throughout Arkansas (*6*). Notably, *R. amblyommii* has been identified as a potential pathogen and is found in lone star ticks (*A. americanum*) (*7*,*8*). We report the presence of DNA consistent with that of *Candidatus* Rickettsia amblyommii and *R. parkeri* in *A. maculatum* ticks in Arkansas.

The Study

We screened 112 *A. maculatum* ticks collected during March 2006–January 2008 from 22 dogs (*Canis lupus familiaris*) and 95 *A. maculatum* ticks collected during the 2008 hunting season from 52 white-tailed deer (*Odocoileus virginianus* Boddaert) for rickettsial DNA. Collectors removed specimens; stored them in vials containing 100% ethanol; and recorded tick collection date, location, and host (*6*). Ticks were identified by species, sex, life stage, and engorgement (*9*). Each sample was bisected longitudinally with a razor blade and subjected to the extraction procedure by using QIAGEN DNeasy (QIAGEN, Valencia, CA, USA) following the manufacturer's protocols.

Tick DNA extracts were screened for SFG *Rickettsia* spp. DNA by PCR by using genus-specific primers for the citrate synthase (*gltA)* (*10*) and rickettsial outer membrane protein B (*rompB*) (*11*) genes. Reaction products were analyzed (*12*), and positive amplicons for *gltA* (513 bp) and *rompB* (578 bp) were sent to the University of North Texas Health Science Center (Fort Worth, TX, USA) for sequence determination. At least 1 amplicon from each host was sequenced to determine the *Rickettsia* species identity. PCR products were hydrolyzed with ExoSAP-IT (USB Corporation, Cleveland, OH, USA), and sequence determination was performed by using a BigDYE Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA) followed by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Inc.) (*13*).

Sequences were edited, aligned, and analyzed with Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) and compared with sequences in GenBank (National Center for Biotechnology Information, Bethesda, MD, USA). BEAST version 1.4.2 software (http://beast.bio.ed.ac.uk/Main_Page) was used to infer phylogenetic relationships and create dendrograms (*14*). The consensus tree ran for 10^6 generations with a burn-in of 2×10^4. Established methods were used (*12*) to conduct parsimony bootstrap and maximum-likelihood analyses. Maximum-likelihood and unweighted parsimony analyses on the alignments were performed by using the branch and bound algorithm of PAUP* 4.0b10 (http://paup.csit.fsu.edu). Outgroup taxa were obtained from GenBank.

Of the 207 ticks, 62 were positive for *Rickettsia* spp. DNA by PCR. Nineteen ticks were positive for *gltA* only, 12 were positive for *rompB* only, and 31 were positive for both genes (Table). Of the ticks collected from white-tailed deer, 28 were positive, and those amplicons were 100% homologous with *Candidatus* Rickettsia amblyommii from GenBank (FJ455415, EU7228827, AY388899) (Table, Figure). Of the positive ticks collected from dogs, 3 had sequences with 100% similarity to either *rompB* (AF123717) or *gltA* (EF102236) of *R. parkeri*. A single tick (unengorged male) had a sequence 98% similar to GenBank sequences EF219464 (*rompB*) and EF451001 (*gltA*). The remaining 30 ticks collected from dogs that were positive all produced amplicons with 100% sequence identity to *Candidatus* R. amblyommii *gltA* (EF450708). However, *rompB* sequences

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Table. Gulf Coast ticks (*Amblyomma maculatum*) collected from white-tailed deer and dogs, Arkansas, USA, 2006–2008*

Source	No. tested	No. (%) positive	Blood meal	*gltA* positive	*rompB*-positive groups				*gltA*- and *rompB*-positive groups					
					2	3	4	5	1	2	3	4	6	NS
White-tailed deer														
Nymph	8	0	Yes											
			No											
Male	46	15 (33)	Yes							2				2
			No	5	2					4				
Female	41	13 (32)	Yes	2	2									
			No	3	3					3				
Total collected from deer	95	28 (29)		10	7	0	0	0	0	9	0	0	0	2
Dogs														
Nymph	8	2 (25)	Yes											
			No	1						1				
Male	95	28 (29)	Yes							1				1
			No	8	1	1	1	1		8	2	3	1	
Female	9	4 (44)	Yes											
			No		1				3					
Total collected from dogs	112	34 (30)		9	2	1	1	1	3	10	2	3	1	1
Total ticks collected	207	62 (30)		19	9	1	1	1	3	19	2	3	1	3

*Of 207 ticks collected, 30% were positive for rickettsiae. Ticks were positive for citrate synthase (*gltA)* gene only, rickettsial outer membrane protein B (*rompB)* gene only, or both genes; *rompB* sequences fell within 6 sequence groups. Ticks were collected from canids during March 2006–January 2008 and from deer during the 2008 deer hunting season. NS, not sequenced.

generated from the same sample set demonstrated greater diversity (Table, Figure).

In total, 3 ticks collected from 3 different canine hosts produced sequences 100% identical to those of *R. parkeri rompB* (AF123717) and *gltA* (EF102236). *Candidatus* R. amblyommii sequences were identified in 29 ticks collected from 13 dogs and 25 ticks collected from 25 deer. The resulting Bayesian tree showed weak support (consistency index 0.792, tree length 159) (Figure). Neighbor-joining and maximum-likelihood trees supported the GenBank homologies.

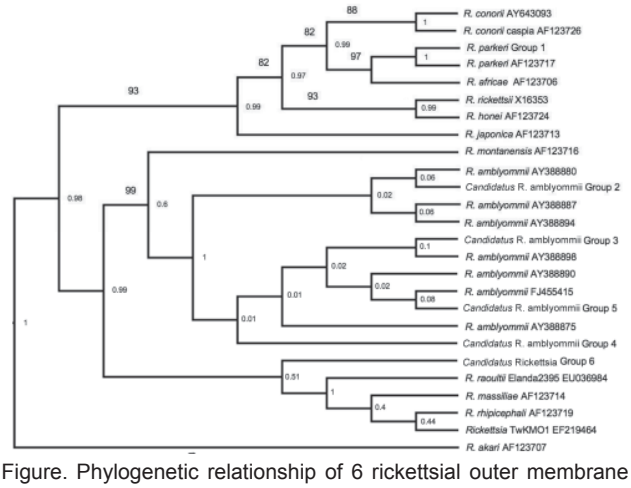

Figure. Phylogenetic relationship of 6 rickettsial outer membrane protein B rickettsiae groups (578 bp) identified in *Amblyomma maculatum* ticks collected in Arkansas and similar rickettsiae identified from GenBank. The tree was constructed by using the maximum-likelihood and maximum-parsimony analysis in BEAST 9 (http://beast.bio.ed.ac.uk/Main_Page) Numbers on lines are bootstrap support values >75 and numbers at nodes are posterior values. Scale bar indicates nucleotide substitutions per site.

Conclusions

We report the identification of SFG rickettsiae in *A. maculatum* ticks collected from Arkansas, specifically *R. parkeri, Candidatus* R. amblyommii, and an uncharacterized *Rickettsia* sp. sequence with high homology to GenBank sequence no. EF219464. Identification of these rickettsiae may be a public health concern given their recent association with cases of spotted fever (*4*,*7*,*8*). The risk for spotted fever transmission to humans is unknown but may be of concern to public health officials in Arkansas because of canid–human relationships and habitat fragmentation that has moved deer ranges closer to human habitation. Additional investigations of the distribution of *A. maculatum* ticks, the pathogenesis of Rocky Mountain spotted fever, and the ticks' relationship to human disease should be conducted.

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Ms Trout is a PhD candidate in the Department of Entomology at the University of Arkansas. Her dissertation focuses on the spatial identification and genetic characterization of ticks and their relationship with *Borrelia* and *Rickettsia* species within Arkansas.

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Bluetongue Virus in Wild Deer, Belgium, 2005–2008

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To investigate bluetongue virus serotype 8 infection in Belgium, we conducted a virologic and serologic survey on 2,416 free-ranging cervids during 2005–2008. Infection emerged in 2006 and spread over the study area in red deer, but not in roe deer.

Bluetongue virus serotype 8 (BTV-8) spread throughout western Europe in 2006. Belgium reported its first case in farm ruminants in 2006. Because some cervid species may be seriously affected by BTV and because they may be reservoir hosts (*1*), we conducted a large-scale survey of BTV-8 in Belgium.

The Study

Postmortem examinations were conducted on 1,620 red deer and 796 roe deer shot by hunters during hunting seasons in 2005–2008 (online Technical Appendix, www.cdc.gov/EID/content/16/5/833-Techapp.pdf). Sex, age, body condition, and macroscopic aspects of hooves, mucosae, and internal organs were recorded. Blood and spleen samples were obtained.

Antibodies against virus protein 7 were detected by using a competition ELISA kit (ID-VET, Montpellier, France). Results were expressed as percentage negativity compared with kit negative control serum, and cutoff values were established. Serum samples from 80 red deer and 40 roe deer were also analyzed by using a virus neutralization (VN) test for BTV-1 and BTV-8. Spleen samples obtained in 2006 and 2007 were used for detection of BTV RNA segment 5 and cellular β-actin transcripts by reverse transcription–quantitative PCR (RT-qPCR) according to a modified procedure of Toussaint et al. (*2*).

To assess performance of the ELISA, we performed receiver operating characteristic analysis. To estimate effects of potential factors (sex and age, year, month and area of sampling) on risk for seropositivity, we used a multivariate logistic regression model. Between-group differences were assessed by using the χ^2 test.

A total of 237 pairs of ELISA and RT-qPCR results from red deer were used for receiver operating characteristic analysis, which yielded an area under the curve of 0.811 and a cutoff value for the ELISA that maximized sensitivity (86%) and specificity (98%). Serologic status was defined as positive (<66%), doubtful (≥66%–≤75%), or negative (>75%) and was comparable to that found for domestic ruminants (*3*). For BTV-8, concordance between ELISA and VN results was 95% for red deer and 82% for roe deer. Neutralizing antibodies to BTV-1 were not detected.

From 2006 on, no gross lesions compatible with bluetongue disease were found. In 2005, all serum samples were negative. For hunting seasons in 2006, 2007, and 2008, seroprevalences were 1.51% (95% confidence interval [CI] 0.89%–2.07%), 52.33% (95% CI 49.91%–54.78%), and 33.95% (95% CI 31.64%–36.26%) for red deer and 2.56% (95% CI 1.43%–3.60%), 2.75% (95% CI 1.62%–3.90%), and 1.67% (95% CI 0.75%–2.51%) for roe deer and showed a significant difference between species ($p<0.0001$ by Cochran-Mantel test).

Yearly profiles of humoral immune responses are shown in Figure 1. Unimodal negatively skewed distributions of percentage negativity in 2005 and 2006 likely reflect seronegative populations. Conversely, bimodal profiles in 2007 and 2008 are compatible with ongoing infections in the red deer population. Spatial evolution of humoral responses in red deer is shown in Figure 2. In 2006, seropositive animals were detected in only 5 districts, of which 4 were the most eastern districts among the 20 sampled; deer in most districts were infected in 2007 (22/25) and 2008 (17/21).

In red deer, multiple logistic regression analysis showed that risk for seropositivity was significantly affected by age (χ^2 84.53, $p<0.0001$), year (χ^2 282.75, $p<0.0001$), and location of sampling (χ^2 63.10, $p<0.0001$), but not by sex (χ^2 0.19, $p>0.90$) or month of sampling (χ^2 2.45, $p>0.45$). Seroprevalence was lower for juveniles than for subadults (odds ratio 2.11, 95% CI 1.47–3.04) and adults (odds ratio 3.79, 95% CI 2.85–4.62). The decrease in 2008 was significant only for juveniles, and the seropositivity gradient decreased toward southern part of the study region (Table). For roe deer, risk for seropositivity was not influenced by any factor.

The 343 spleen samples (230 red deer and 113 roe deer) tested by RT-qPCR in 2006 and the 193 samples (roe deer) tested in 2007 were negative for BTV RNA. Conversely, ≈14% (33/237) of red deer β-actin–positive samples (237/331) were positive for BTV RNA (online Technical Appendix). These 33 animals did not have gross lesions suggestive of bluetongue disease. Of 32 serum sam-

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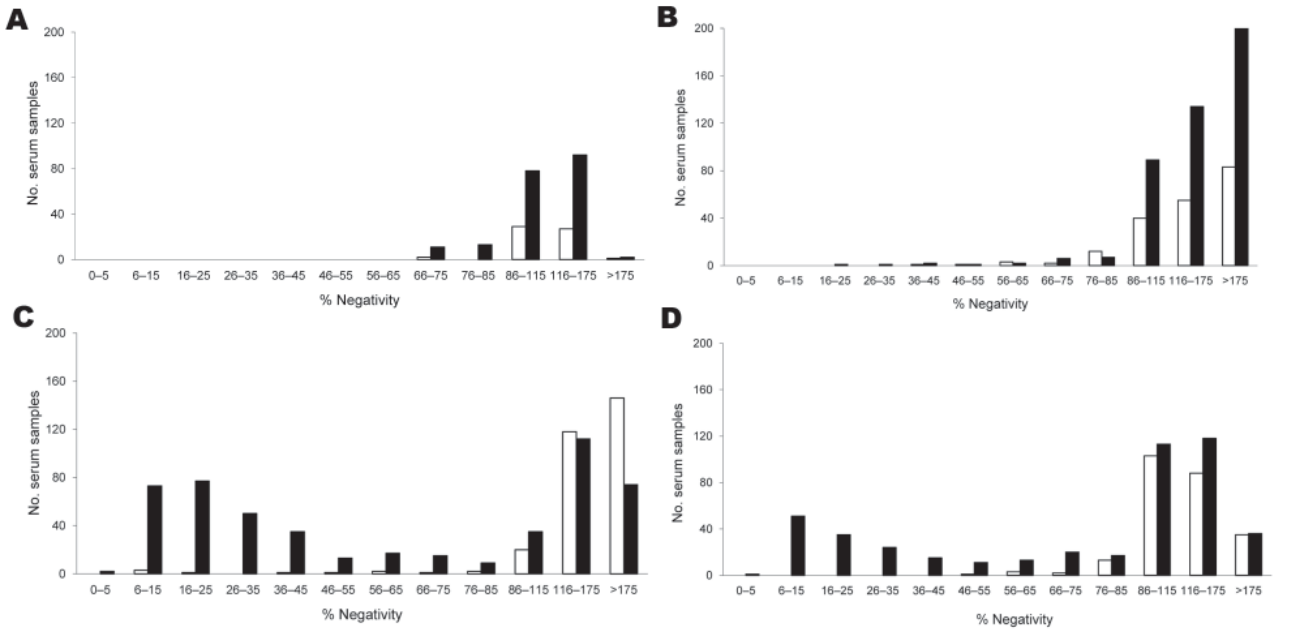

Figure 1. Frequency distribution of results of a competitive ELISA for detecting antibodies against bluetongue virus in serum samples from roe deer (white columns) and red deer (black columns) during the hunting seasons of A) 2005, B) 2006, C) 2007, and D) 2008, Belgium. Hunting was conducted in 30 (area 12,851 km^2) of 37 (area 16,844 km^2) forest districts known to contain wild cervids. The study population of wild cervids in southern Belgium (49°30′N–50°48′N) is estimated to be ≈11,000 red deer (*Cervus elaphus*) and ≈33,000 roe deer (*Capreolus capreolus*). Serum samples with a percentage negativity value (relative to the negative control serum) <66 were considered positive.

ples available, 26 were seropositive, 1 was doubtful, and 5 were seronegative, which suggested that these animals had been infected recently. Two pan-BTV RNA–positive spleen fragments, sampled at the end of hunting season in 2008, were reassessed by using a BTV-8 genotype–specific RT-qPCR (*4*); results were positive for all.

Conclusions

Our study provides evidence that BTV-8 infects wild cervid populations in Belgium. For red deer, a few infections occurred in 2006 in the eastern part of Belgium, i.e., the area in which the ovine and bovine cases had been detected (*5*). In 2007, the infection spread west and southwest, and its seroprevalence increased. In 2008, distribution remained stable but overall seroprevalence decreased, mostly among juveniles. Distribution profiles of antibodies against BTV in 2007 and 2008 showed a bimodal profile. A large number of serum samples showed percentage negativity values between positive and negative values, which is indicative of ongoing virus transmission by vector midges during the hunting season. More animals were infected in eastern and central than in southern Wallonia, a finding that resembles the spatial distribution of the virus in livestock and might be correlated with lower density of cattle populations and cooler temperatures in hilly southern districts (*6*).

The proportion of seropositive animals increased with age, probably resulting more from prolonged exposure of adults to the vector, than to any resistant status of juveniles. Decreasing overall seroprevalence in 2008 might be caused by mandatory vaccination of domestic ruminants and spontaneously acquired herd immunity within the red deer population, which reduced virus prevalence among insect vector populations in 2008. Because seropositive subadults and adults sampled in 2008 could have been infected in 2007, seroprevalence among juveniles should more accurately reflect the level of exposure to infected insect vectors. If this explanation was true, the decrease in seroprevalence among juveniles in 2008 would confirm reduction of the virus insect reservoir.

Although red deer and roe deer samples were collected at the same locations and during the same hunting events, seroprevalence was lower among roe deer. Because 5 of 12 ELISA-positive and none of the ELISA-negative roe deer serum samples were positive by BTV-8 VN test and a similar between-species pattern was found by RT-qPCR, poor sensitivity of the ELISA in roe deer as the cause of between-species difference can be ruled out. Host-related and vector-related factors might account for this difference. Because red deer live in large groups and move more, they might be more exposed to insects/pathogens than are roe deer, which live in small groups in winter and have smaller

Figure 2. Distribution of red deer samples obtained in Belgium (Wallonia) in A) 2005, B) 2006, C) 2007, and D) 2008, and location of forest districts. White circles indicate districts where only seronegative animals were detected, and black circles indicate districts where seropositive animals were detected. Scale bar indicates 100 km.

home-range sizes and are seasonally territorial (*7*). Moreover, a recent follow-up of *Culicoides* spp. midge feeding patterns reported variations in host attractiveness, which could correlate with exhaled carbon dioxide and acetone (*8*), specific phenolic compounds emitted from urine (*9*) or hair fragrance (*10*).

BTV might be maintained in an as yet unknown reservoir host population with a long or relapsing viremia and in which clinical signs are absent. Because no excess illness or death occurred in 2007–2008, BTV-8 infection of wild cervids is probably benign enough to go unnoticed. Some spleen samples from dead red deer found during winter also showed positive results by RT-qPCR even if BTV was not the cause of death (A. Linden, unpub. data). Coupled with the high seroprevalence we report, the possibility that red deer are BTV reservoirs warrants further investigation.

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Table. Seroprevalence of bluetongue virus among red deer, by age and study area, Belgium, 2006–2008*

Characteristic	2006		2007		2008	
	No. positive/ no. tested (%)	95% CI	No. positive/ no. tested (%)	95% CI	No. positive/ no. tested (%)	95% CI
Age†						
Adults	4/221 (1.81)	0.05–3.57	142/216 (65.74)	59.41–72.07	111/185 (60.00)	52.94–67.06
Subadults	1/59 (1.69)	0.00–4.99	45/82 (54.88)	44.11–65.65	27/75 (36.00)	25.14–46.86
Juveniles	2/178 (1.12)	0.00–2.67	80/213 (37.56)	31.06–44.06	11/191 (5.76)	2.46–9.06
Area‡						
Eastern	ND	ND	81/161 (50.31)	42.59–58.03	43/123 (34.96)	26.53–43.39
Central	ND	ND	103/154 (66.88)	59.45–74.32	61/135 (45.19)	36.79–53.58
Southern	ND	ND	32/105 (30.48)	21.67–39.28	27/133 (20.30)	13.46–27.14

*CI, confidence interval; ND, not determined.
†Adults, >2 y of age; subadults, 1–2 y of age; juveniles, <1 y of age.
‡Fourteen forest districts were distributed among 3 nonadjacent areas, 5 in the eastern area, 4 in the central area, and 5 in the southern area.

Dr Linden is a professor in the Department of Infectious and Parasitic Diseases in the Veterinary Faculty of the University of Liège. Her research interests focus on wildlife diseases.

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Nosocomial Outbreak of Crimean-Congo Hemorrhagic Fever, Sudan

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To confirm the presence of Crimean-Congo hemorrhagic fever in Sudan, we tested serum of 8 patients with hemorrhagic fever in a rural hospital in 2008. Reverse transcription–PCR identified Crimean-Congo hemorrhagic fever virus. Its identification as group III lineage indicated links to virus strains from South Africa, Mauritania, and Nigeria.

Crimean-Congo hemorrhagic fever virus (CCHFV; family *Bunyaviridae*, genus *Nairovirus*) is a tick-borne virus. Its tripartite RNA genome consists of small (S), medium, and large segments. The virus is distributed throughout much of Africa, Asia, and southern Europe (*1*–*5*). In some regions, the virus is responsible for annual outbreaks of hemorrhagic fever with high case-fatality rates; in others, it causes sporadic cases only. Because of its association with rapid-onset hemorrhagic fever and an ≈30% case-fatality rate, CCHFV is on the US Select Agent list of agents considered to have bioterrorism potential (*2*–*5*).

Distribution of CCHF largely mirrors that of its Ixodid tick hosts, particularly those of the genus *Hyalomma* (*1*). Persons become infected when bitten by virus-infected ticks or after contact with blood or tissue from viremic livestock or other persons. Outbreaks often involve persons in rural communities, such as shepherds, slaughterhouse workers, or medical staff of resource-poor hospitals. Despite presence of *Hyalomma* tick vectors in Sudan, no CCHF cases have been confirmed there. However, in the past 2 years, suspected CCHF outbreaks and sporadic cases in the Kordufan region of Sudan have been reported.

From a public health perspective, confirming CCHF in Sudan and determining which virus lineages may be present in this region will provide a more detailed understanding of the movement of virus strains and identification of areas at risk for CCHFV. We therefore analyzed an outbreak of hemorrhagic fever, including a nosocomial chain of transmission in a rural hospital in Sudan in 2008.

The Study

In October 2008, an outbreak of hemorrhagic fever was reported in Al-fulah, Kordufan, Sudan. The index patient was a 60-year-old man who had worked as a butcher. The source of his infection was suspected to have been tissues and blood of an infected animal, although follow-up investigation was unable to precisely determine the source. He was admitted to a rural hospital with an acute febrile hemorrhagic illness after 3 days of high fever, chills, and headache. He had taken antimalarial medication at home, but his condition did not improve. He had epistaxis, black bloody vomitus, and diarrhea on the last 2 days of his illness. He died on day 5 after onset of illness.

No protective gloves or antiseptic products were available at the hospital. Illness developed in a male nurse who had provided care to the index patient 6 days after the index patient had been admitted to the hospital and in the chief male nurse a few days after that. The index patient's sister was also considered to have a suspected case; she had sought care at the hospital after a heavy menstrual period that progressed to massive vaginal bleeding. The midwife who performed the gynecologic examination later became ill with high fever, vomiting of blood, and bloody diarrhea. As is tradition and social obligation in rural hospitals in this region, 2 relatives of the index patient had alternated caring for him (e.g., dressing him, changing his mattresses and bed sheets, nursing, and sleeping beside him) while he was in the hospital, and both acquired the infection (rapid onset of fever, headache, nausea, vomiting of blood, and bloody diarrhea). No details were available for 3 other patients with hemorrhagic fever associated with the hospital.

Of these 10 patients, 9 were admitted to a rural hospital in Al-fulah, where 6 continued to bleed, subsequently became comatose, and died. Records were unavailable for the other 3. In addition, 3 probable cases in the community were reported. Each of these 3 persons had a course of hemorrhagic disease and death that was compatible with CCHF; they had not been admitted to the hospital and could not be traced because of poor security conditions in the region. Patient ages varied from 15 to 70 years. Nosocomial transmission of the virus was likely the result of lack of personal protection for the hospital staff and the attending relatives, as has been often noted during previous outbreaks (*6*).

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Of the patients for whom serum samples were available, 8 had evidence of acute CCHFV infection. Direct immunofluorescence assay detected no antibodies to CCHFV in any of the serum samples; however, all samples had been collected on days 1–3 of illness. Virus RNA extracted from each of the samples by QIAamp (QIAGEN GmbH, Hilden, Germany) was positive according to reverse transcription–PCR (RT-PCR) specific for CCHFV (*7*). The RNA was then used in RT-PCRs to amplify the entire virus S segment for complete sequencing as described (*8*). The full-length S segment nucleotide sequence of the strains from Sudan was 1,673 nt long, and the 8 viral sequences were identical with the exception of that from patient 4 (GenBank accession nos. GQ862371–2). A maximum-likelihood phylogenetic analysis of the S segment sequences placed the viruses from Sudan in group III (*8*), which is composed exclusively of viruses of African origin, including South Africa, Mauritania, and Nigeria (Figure). The highest nucleotide sequence identity was seen with strains from South Africa.

Conclusions

Laboratory confirmation of 8 cases of CCHF demonstrates the presence of this disease in Sudan. Genetic analysis of the viruses showed that the strain involved was similar to strains found in South Africa, Mauritania, and Nigeria.

Detailed analysis of virus outbreaks is often limited by the lack of appropriate high-containment facilities required for virus isolation. However, appropriately sampled and stored acute-phase serum samples can have high titers of the virus, which enable extraction of virus RNA and genome sequencing studies without prior amplification of the virus in cell culture. In this study, serum specimens from 8 patients who died were positive for CCHFV by RT-PCR. Lack of virus-specific antibodies 1–3 days after onset of illness can be explained by the severity and rapid course of the disease, which does not allow sufficient time for antibody production. Virus-specific antibodies are, however, generally seen later in the course of illness and in persons who survive the infection.

Antibody studies have suggested the presence of various arboviruses in Sudan (*10*,*11*). Indirect evidence for CCHFV in animals in Sudan came from finding CCHFV-specific antibodies in animals imported from Sudan: camels in Egypt (*12*) and sheep and goats in Saudi Arabia (*13*). The finding that the Al-fulah outbreak was caused by a CCHFV strain from genetic group III illustrates how different virus strains and lineages can move with livestock transport or possibly bird migrations. The genome plasticity of the virus is surprisingly high for an arthropod-borne virus. This genetic diversity appears to be the result of not only accumulation of mutations but also of frequent RNA segment reassortment and even RNA recombination (*8*,*14*,*15*).

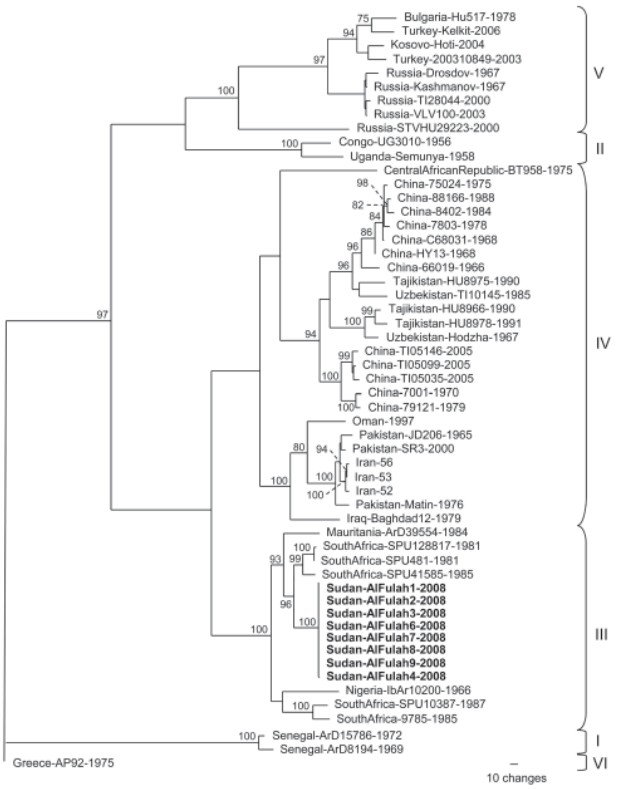

Figure. Phylogenetic relationship of Crimean-Congo hemorrhagic fever virus (CCHFV) full-length small (S) segments. Phylogenetic analysis used 47 full-length CCHFV S segments available in GenBank. GARLI (v0.96b8) (*9*) with default settings was used to generate a maximum-likelihood tree with bootstrap support values from 1,000 replicates. From the analysis, a 50% majority-rule tree was constructed. Virus strains from Sudan patients 1, 2, 3, 6, 7, 8, and 9 (GenBank accession no. GQ862371) were identical, and the virus sequence from patient 4 (GenBank accession no. GQ862372) differed by 1 nt. Each strain is listed by its location, strain name, and year of isolation, when available. **Boldface** indicates strains from Sudan; braces indicate previously described genetic lineages (*8*).

Clearly, this CCHFV is present in Sudan, and physicians should consider CCHF as a diagnosis for hemorrhagic fever cases in the region. In addition, efforts to provide appropriate personal protective supplies and training to medical staff in rural areas should be increased to help minimize risk for caregivers.

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etymologia

Tropheryma whipplei

[tro-fer'ĭ-mə wi'-pəl-ē-ī]

The genus name of this gram-positive, rod-shaped, soil-dwelling bacterium was taken from Greek *trophe* (nourishment) and *eryma* (barrier) because malabsorption was a feature of the infection it caused. The species name honors George Hoyt Whipple (1878–1976), an American pathologist and medical educator, who, in 1907, first described the clinical syndrome later known as Whipple's disease. In 1991, when sections of the genome were sequenced, the organism was named *T. whippelii*; the spelling was corrected in 2001.

Source: La Scola B, Fenollar F, Fournier P-E, Altwegg M, Mallet M-N, Raoult D. Description of Tropheryma whipplei gen. nov., sp. nov, the Whipple's disease bacillus. Int J Syst Evol Microbiol. 2001;51:1471–9.

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Extensively Drug-Resistant Tuberculosis, Burkina Faso

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Because data from countries in Africa are limited, we measured the proportion of extensively drug-resistant (XDR) tuberculosis (TB) cases among TB patients in Burkina Faso for whom retreatment was failing. Of 34 patients with multidrug-resistant TB, 2 had an XDR TB strain. Second-line TB drugs should be strictly controlled to prevent further XDR TB increase.

Extensively drug-resistant (XDR) tuberculosis (TB) represents an emerging public health problem worldwide, characterized by alarming fatality rates regardless of patients' HIV status (*1*,*2*). XDR TB is defined as in vitro resistance to isoniazid and rifampin plus any fluoroquinolone and at least 1 injectable drug (capreomycin, kanamycin, or amikacin).

Since 2006, a total of 49 countries have reported XDR TB (*3*). Data from countries in Africa are scant, with the exception of South Africa, where the prevalence is high, especially among HIV-infected persons (*3*). However, these data may reflect diagnostic limitations rather than the true epidemiologic situation. In Burkina Faso, a low-income country in western Africa, TB prevalence is 226 new cases per 100,000 population (*4*), and multidrug-resistant (MDR) TB is estimated at 2.1% among new patients with smear-positive TB (*5*). During a systematic search for MDR TB among TB patients in Burkina Faso for whom a retreatment regimen was failing, we documented 2 cases of XDR TB.

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The Study

In 2006, a program was established by the National Tuberculosis Program, Burkina Faso; the University of Brescia, Italy; and the San Raffaele Scientific Institute, Italy, to perform drug-susceptibility testing (DST) on sputum samples from patients listed in the Burkina Faso Chronic TB Register. To be included in this register, patients must have experienced treatment failure after standard and retreatment regimens consisting of the following (in order): 2 months of streptomycin, rifampin, isoniazid, pyrazinamide, and ethambutol; then 1 month of rifampin, isoniazid, pyrazinamide, and ethambutol; and then 5 months of rifampin, isoniazid, and ethambutol. Failure was defined as a persistently positive sputum smear after 5 months of treatment. All patients were informed and consented to the study.

From January 2006 through March 2009, a total of 156 patients with chronic TB were registered in Burkina Faso. For 88 patients, sputum samples were collected before treatment with second-line drugs; for 48, they were collected at 1–12 months of treatment with second-line drugs. Samples were immediately frozen and stored at –20°C before being transferred on dry ice for culture and first-line DST at the University of Brescia and for second-line DST and genotyping at San Raffaele Scientific Institute. Samples were cultured on an MGIT 960 automated system (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) according to the manufacturer's instructions. DST to first- and second-line drugs was performed on all *Mycobacterium tuberculosis* isolates by classic dilution method.

M. tuberculosis was isolated from 50 samples; 45 patients had not yet taken second-line drugs and 5 had. Of the 50 isolates, 34 (68%) were confirmed as MDR TB, 29 from untreated patients and 5 from treated patients. We identified 2 cases of XDR TB (5.9% of all MDR TB cases): 1 patient was sampled at month 24 of treatment with second-line drugs, and 1 was initially classified as having MDR TB before his disease progressed to XDR TB during follow-up treatment with second-line drugs.

Patient 1 was a 33-year-old man who was born in Côte d'Ivoire, moved to Burkina Faso in 2000, and received a diagnosis of smear-positive pulmonary TB in July 2003. He received an 8-month standard treatment regimen (including 6 months of continuation with isoniazid and ethambutol). Treatment was directly observed during the first 2 months. In January 2004, his treatment was classified as failed, and he immediately started a standard retreatment regimen. Because his sputum did not clear by month 5, the patient was registered as a patient with chronic TB. During 2004, he traveled throughout Mali and Côte d'Ivoire. Back in Burkina Faso in January 2005, he was admitted to the reference national hospital for patients with chronic TB. His HIV test result was negative. At this time neither culture and DST nor a standard second-line drug regimen approved by the

Green Light Committee were available in Burkina Faso. The patient was empirically prescribed kanamycin, ethionamide, ciprofloxacin, and pyrazinamide, which he took under direct observation as a hospital inpatient for 21 months; at discharge, his sputum samples remained positive.

In December 2006, 2 sputum samples showed resistance to all first-line drugs; resistance to second-line drugs amikacin, ofloxacin, ethionamide, and cycloserine; and susceptibility to para-aminosalicylic acid and capreomycin. Appropriate and effective drugs were unavailable in the country. The patient was readmitted to the ward for patients with chronic TB and placed in a single isolation room. He died of TB in August 2008. Known contacts were monitored closely. A housemate was listed in the Chronic TB Register in June 2006. He traveled from Burkina Faso to Israel before DST could be performed. The patient's sister-in-law died of MDR TB in March 2007; DST confirmed resistance to all first-line drugs but susceptibility to amikacin and ofloxacin.

Patient 2 was a 44-year-old man. His MDR TB was diagnosed in Côte d'Ivoire in June 2007, and he immediately moved back to Burkina Faso to seek care. His HIV test result was negative. Sputum samples collected in July 2007 grew MDR *M. tuberculosis* resistant to amikacin, kanamicin, ethionamide, and closerine but sensitive to ethambutol and ofloxacin. In September 2007, the patient started treatment with Green Light Committee–approved second-line drugs (6 months of pyrazinamide, ofloxacine, kanamycin, ethionamide, and cycloserine followed by 15 months of ofloxacin, ethionamide, and cycloserine). Treatment was directly observed during the first 13 months. His sputum samples remained positive throughout treatment, and additional DST in October 2008 showed an XDR strain of *M. tuberculosis*. Mycobacterial interspersed repetitive unit genotyping showed an identical pattern for strains detected initially and during follow-up.

Conclusions

Our findings confirm that XDR TB can be found wherever the search capacity exists. Thus, despite unavailability of evidence for the widespread existence of drug resistance, high priority should be given to strengthening laboratory capacities in sub-Saharan Africa (*6*). Also, because XDR TB developed while patient 2 was receiving second-line TB drugs, optimal adherence during intensive and continuation phases of second-line treatment regimens should be ensured. Staff should receive specific training with regard to managing the frequent side effects.

Our study had 1 major limitation. Because our sample was a select population and included patients already receiving treatment with second-line drugs, we may have overestimated the proportion of XDR TB cases among MDR TB cases.

Mechanisms of emergence of XDR TB in Burkina Faso differ from those in South Africa, where most identified XDR TB cases are primarily resistant, occur among HIV-infected patients, and result from exposure in the hospital or the community (*7*). Because each of the 2 XDR TB patients in Burkina Faso reported long-term stays in neighboring countries, we believe that response to the MDR TB challenge should be based on regional rather than national strategies.

Our study supports current policies for strictly controlled introduction of second-line drugs and the current Green Light Committee strategy that requires demonstration of compliance with guidelines for proper management of MDR TB before granting access to second-line drugs (*8*). Stakeholders and TB program managers in Africa should be reminded that the main reasons for development of resistance in TB are poor patient adherence to treatment regimen, inappropriate drug prescription, irregular drug supply, and poor drug quality (*9*). When these factors occur with use of second-line TB drugs, the result will be XDR TB.

Rapid adoption of a programmatic approach to management of MDR TB (*6*) is warranted in Burkina Faso and probably other countries in western Africa. In countries without effective programs for community or outpatient care of patients with MDR TB, hospital care in reference centers enables appropriate follow-up during the initial phases of treatment. In this context, implementation of appropriate infection control measures should rank high among program priorities (*10*).

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Streptococcus dysgalactiae subsp. *equisimilis* Bacteremia, Finland, 1995–2004

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We conducted a retrospective population-based study of 140 episodes of *Streptococcus dysgalactiae* subsp. *equisimilis* bacteremia occurring in Finland during 1995–2004. Rare *emm* types were associated with more severe disease and increased mortality rates. Skin and soft tissue infections were more frequent clinical signs among cases caused by common *emm* types.

Lancefield groups C and G β-hemolytic streptococci (GCS and GGS) may colonize the pharynx, skin, gastrointestinal tract, and female genitourinary tracts (*1*). According to recent taxonomic studies, large colony-forming groups C and G streptococci that infect humans are classified as *Streptococcus dysgalactiae* subsp. *equisimilis* (*2*). *S. dysgalactiae* subsp. *equisimilis* and *S. pyogenes* share virulence factors (*3*,*4*). The M protein is an important virulence factor because it confers resistance to phagocytosis (*5*). As with *emm* genes of *S. pyogenes*, the *emm* homologs of groups C and G *S. dysgalactiae* subsp. *equisimilis* are used for sequence-based typing (*4*,*6*,*7*), with >50 sequence types currently described (www.cdc.gov/ncidod/ biotech/strep/emmtypes.htm). The aim of our study was to determine the clinical signs, epidemiologic characteristics, and *emm* types of *S. dysgalactiae* subsp. *equisimilis* bacteremia during the 10-year observation period in Finland.

The Study

We retrospectively reviewed the medical records of all adult patients (>16 years of age) in Pirkanmaa Health District, Finland, with ≥1 blood cultures positive for group C or group G *S. dysgalactiae* subsp. *equisimilis* from January 1995 through December 2004. The Pirkanmaa Health District (460,000 inhabitants) has 1 tertiary care hospital (Tampere University Hospital) and 4 other hospitals (Hatanpää City Hospital and the District Hospitals in Valkeakoski, Vammala, and Mänttä). Laboratory records were screened to identify all blood cultures positive for group C or group G *S. dysgalactiae* subsp. *equisimilis* during the study period. Our case definition included all patients who had a positive blood culture for *S. dysgalactiae* subsp. *equisimilis* and clinical signs compatible with septicemia. A severe disease was defined as a septicemia leading to death or needing intensive care unit treatment. All 128 GGS isolates and 12 of 18 GCS isolates were confirmed to be *S. dysgalactiae* subsp. *equisimilis*. Thus, these 140 episodes of *S. dysgalactiae* subsp. *equisimilis* septicemia (involving 137 patients) comprised the present study. Two of the isolates (1 GGS and 1 GCS) were not available for *emm* typing, and 138 of the *S. dysgalactiae* subsp. *equisimilis* isolates (from 135 patients) were sequenced to identify the *emm* gene.

Routine blood samples were drawn into aerobic and anaerobic bottles and cultivated by standard methods as reported (*8*). *S. dysgalactiae* subsp. *equisimilis* isolates were further analyzed by *emm* typing. Nontypeable strains and strains isolated from patients with recurrent bacteremia were characterized by using pulsed-field gel electrophoresis (PFGE).

The *emm* typing was performed according to the protocol of the Centers for Disease Control and Prevention (www.cdc.gov/ncidod/biotech/strep/strepblast.htm). If the *emm* gene could not be amplified with primers 1 and 2, alternative primers MF1/MR1 were used (*9*). PFGE was performed as described (*10*). DNA profiles were analyzed by using Bionumerics software (Applied Maths, Kortrijk, Belgium) and interpreted according to the guidelines described (*11*). Strains with >85% similarity were considered to be related types.

SPSS software version 7.5 (SPSS, Chicago, IL, USA) was used for statistical analyses, and a 2-sided p value <0.05 was regarded as the level for significance. Categorical data were analyzed by χ^2 test or Fisher exact test as appropriate. Nonparametric data were analyzed by using the Mann-Whitney U test. Odds ratios were expressed with 95% confidence intervals.

The median age of patients (73 men, 62 women) was 67 years (range 17–90 years). Cardiovascular diseases (41%), diabetes (25%), and malignancies (23%) were the 3 most prominent underlying conditions. We found 18 *emm* types (including 4 subtypes of stG6: stG6.0, stG6.1, stG6.3, and stG6.4). StG480 (27 isolates), stG6 (23 isolates), and stG485 (22 isolates) were the 3 most common *emm* types and represented 51% of all isolates (Figure 1). Eight of group G *S. dysgalactiae* subsp. *equisimilis* isolates remained nontypeable. PFGE analysis showed 2 strains to be related (>85% similarity). The rest of the nontypeable strains were sporadic (6 isolates).

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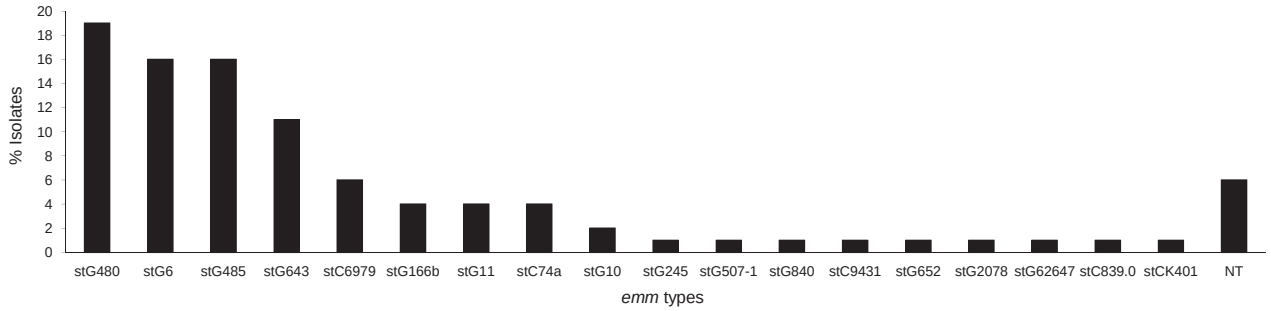

Figure 1. *emm* types of 138 *Streptococcus dysgalactiae* subsp. *equisimilis* bacteremic isolates obtained during 1995–2004, Finland. NT, nontypeable.

We divided bacteremia episodes into 2 groups: those caused by the 5 most common *emm* types and each representing >5% of all episodes (97 episodes, common *emm* types) and those caused by the less common or nontypable *emm* types (41 episodes, rare *emm* types). We could not find an association between *emm* type and clinical features such as age or underlying disease. Severe disease was caused more often by rare *emm* types than by common *emm* types. Mortality rates were higher in patients with bacteremia caused by rare types than that caused by common types (Table 1). Four patients had recurrent *S. dysgalactiae* subsp. *equisimilis* bacteremia (Table 2). PFGE profiles showed that strains isolated from the same patient in recurring infections were identical (Figure 2).

Common *emm* types were more frequently manifested as skin and soft tissue infections than were rare *emm* types, 75% vs. 54%, respectively (p = 0.012). The most frequent source of bacteremia was cellulitis (51%). We also found an association between a common *emm* type and cellulitis. Cellulitis was a more frequent clinical sign among patients with infections caused by common *emm* types than by rare *emm* types (p = 0.007); 64% of patients infected by common *emm* types had cellulitis as an initial clinical manifestation versus 39% of patients infected by rare *emm* types.

Conclusions

Our study showed that mortality rates were higher in patients with *S. dysgalactiae* subsp. *equisimilis* bacteremia caused by rare *emm* types than in those with bacteremia caused by common *emm* types. The reason for this finding is unclear. One explanation for this might be that patients contract certain prevailing bacterial strains (so-called common types) more often, and a prior antigen challenge and subsequent humoral response may play a role. Severe disease (death or intensive care unit treatment) was also caused more often by rare *emm* types than by common *emm* types. We found also an association between a common *emm* type and cellulitis as a clinical manifestation; the common *emm* types were also associated with skin and soft tissue infections.

In our comprehensive study with molecular typing data for 138 invasive *S. dysgalactiae* subsp. *equisimilis* isolates from human infections, we found 18 *emm* types, which is consistent with previous reports by Cohen-Poradosu et al. (*12*) and Broyles et al. (*13*) . These 2 studies reported stG485.0. or StG6, StG245, and StG2078 as the most common *emm* types, respectively. Thus, *emm* typing provides a useful tool for comparative epidemiologic analysis of GGS isolates from various geographic regions. Our results also suggest that certain *emm* types may prevail among bacteria

Table 1. Disease severity among 138 episodes of *Streptococcal dysgalactiae* subsp. *equisimilis* bacteremia, Finland, 1995–2004*

Disease severity	No. (%) common *emm* types, n = 97	No. (%) rare *emm* types, n = 41	Odds ratio (95% CI)	p value†
30-day mortality rate	11 (11)	12 (29)	3.2 (1.3–8.1)	0.01
Patient admitted to ICU	5 (5)	6 (15)	3.2 (0.9–11)	0.084
Patient death or ICU treatment	12 (12)	15 (37)	4.1 (1.7–9.8)	0.001
Hypotension‡	13 (13)	10 (24)	2.1 (0.8–5.2)	0.113
DIC§	2 (2)	6 (15)	8.1 (1.6–42.3)	0.009
Multiorgan failure	2 (2)	4 (10)	5.1 (0.9–29.2)	0.064
STSS¶	2 (2)	4 (10)	5.1 (0.9–29.2)	0.064

*CI, confidence interval; ICU, intensive care unit; DIC, disseminated intravascular coagulation; STSS, streptococcal toxic shock syndrome. Patients who had both clinical data and isolates available.
†χ^2 test or Fisher exact test as appropriate.
‡Hypotensive (BP <90 mm Hg) at least once 0–2 days after positive blood culture.
§Thrombocyte count <100 x 10^9/L.
¶The definition of STSS was based on a consensus definition, including identification of β-hemolytic streptococci from a normally sterile site, septic shock, and multiorgan failure.

Table 2. Characteristics of recurrent episodes of group G *Streptococcal dysgalactiae* subsp. *equisimilis* bacteremia, Finland, 1995–2004*

Patient no.	*emm* type			Time to recurrence, mo	Clinical signs	PFGE pattern
	Episode 1	Episode 2	Episode 3			
1	stG6	stG6	stG6	15; 3	Cellulitis	Unique, identical in episodes 1–3
2	stG6	stG6		68	Cellulitis	Unique, identical in episodes 1 and 2
3	stG480†	stG480		28	Spondylitis	Unique, identical in episodes 1 and 2
4	stG480	NA‡		21	Cellulitis	Unique

*PFGE, pulsed-field gel electrophoresis.
†Blood culture taken outside Pirkanmaa Health District, isolate available.
‡Blood culture taken outside Pirkanmaa Health District, no isolate available.

that cause human infections. Our study did not show any obvious time shifts in the occurrence of certain *emm* types.

A noteworthy finding in our series was the high frequency of recurrent group G *S. dysgalactiae* subsp. *equisimilis* bacteremia as reported earlier (*12*,*14*). Clinicians should be alert to this phenomenon, which seems to be more common than recurrent group A streptococcal bacteremia.

The dynamics of interspecies transfer of virulence loci between group A streptococci, GGS, and GCS (*3*), as well as potential genetic transfer or intragenomic events causing interconversion of group antigen types, remains to be resolved. Further characterization of the strains by multilocus sequence typing would be of interest (*15*).

We conclude that severity of disease and mortality rates were higher in persons with *S. dysgalactiae* subsp. *equisimilis* bacteremia caused by rare *emm* types than that caused by common *emm* types. Skin and soft tissue infections such as cellulitis were significantly more frequent clinical signs among episodes caused by common *emm* types.

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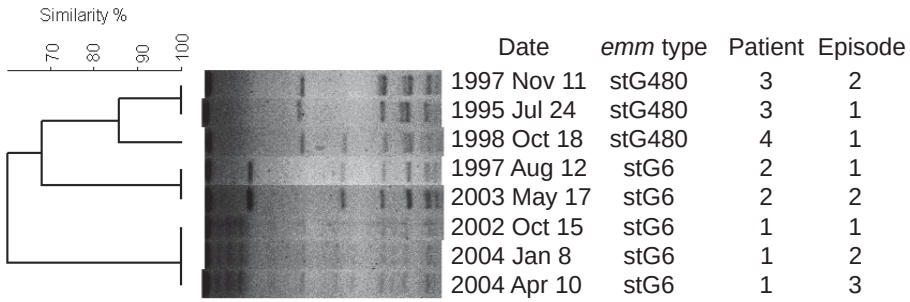

Figure 2. Dendrogram and pulsed-field gel electrophoresis (PFGE) profiles of the strains isolated from patients with recurrent group G *Streptococcus dysgalagtiae* subsp. *equisimilis* bacteremia, Finland. Dendogram was generated by using Bionumerics software (Applied Maths, Kortrijk, Belgium) with a 1.0% lane optimization and 1.5% band position tolerance.

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Dengue Virus Surveillance for Early Warning, Singapore

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In Singapore, after a major outbreak of dengue in 2005, another outbreak occurred in 2007. Laboratory-based surveillance detected a switch from dengue virus serotype 1 (DENV-1) to DENV-2. Phylogenetic analysis showed a clade replacement within DENV-2 cosmopolitan genotype, which accompanied the predominant serotype switch, and cocirculation of multiple genotypes of DENV-3.

Dengue poses a threat to public health in >100 countries worldwide. Despite improvements in diagnostics and clinical management, the number of dengue cases continues to rise globally; 2.5 billion persons are at risk for infection (*1*). Dengue virus (DENV) belongs to the genus *Flavivirus* and contains a positive-strand RNA genome that encodes 3 structural proteins—core protein, membrane-associated protein, envelope protein—and 7 nonstructural proteins. DENV consists of 4 genetically and antigenically distinct serotypes, 1–4.

Singapore has seen a resurgence of dengue cases since the late 1980s, after 2 decades of successful control that relied mostly on an integrated vector-control program (*2*,*3*). The recent epidemiology of dengue in Singapore is characterized by a 5–6-year cycle; incidence rates increase within each cycle before collapsing into 1 or 2 lull years. During an unprecedented dengue outbreak in 2005, a total of 14,006 cases and 27 deaths were reported (*4*). The outbreak was associated with a switch in predominant serotype, from DENV-2 to DENV-1, in 2004 (*5*,*6*). In 2007, Singapore experienced another dengue outbreak after a lull in 2006. We report the laboratory and surveillance findings that assisted vector-control operations during the 2007 dengue outbreak in Singapore.

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The Study

In Singapore in 2005, as part of an integrated vector-control program, laboratory-based dengue virus surveillance was established for close monitoring and investigation of the circulating dengue virus serotypes. Samples were sent to the Environmental Health Institute from Tan Tock Seng Hospital, which cares for ≈40% of all reported dengue patients in Singapore, and from a network of participating general practitioners throughout the country. PCR to detect dengue virus RNA and serotyping were performed at the Environmental Health Institute according to its in-house real-time PCR protocol (*7*). The numbers of dengue-positive samples serotyped were 186 in 2006, 889 in 2007, and 918 in 2008, and represent ≈10% of the total dengue cases reported each year by the Ministry of Health.

The envelope protein gene of DENV (≈1,480 nt) was amplified by reverse transcription–PCR and directly sequenced by using an automated DNA sequencer (ABI 3100; Applied Biosystems, Foster City, CA, USA). Sequences were aligned and submitted to GenBank (accession nos. GQ357666–892). Phylogenetic analysis of DENV sequences was conducted by using the maximum-likelihood method as implemented in PAUP* software, version 4.0b10 (*8*), and compared with sequence data obtained from GenBank.

During 2006–2008, all 4 DENV serotypes were detected (Figure 1). DENV-1 (21.7%) and DENV-2 (69.3%) were the predominant serotypes throughout the study period; DENV-3 (7.8%) and DENV-4 (1.2%) were less prevalent. In 2006, the number of DENV cases was relatively low, and DENV-1 remained the predominant serotype after the major 2004–2005 outbreak. During January–September 2006, 75%–100% of samples collected each month contained DENV-1. In early January 2007, the predominant circulating serotype switched from DENV-1 to DENV-2.

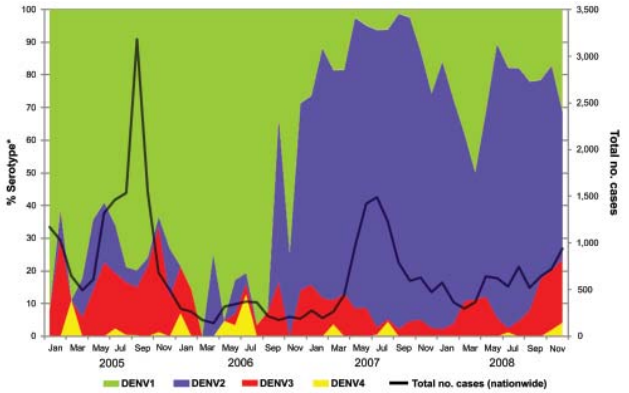

Figure 1. Trends of monthly dengue cases in Singapore, 2005–2008, showing a switch in predominant serotype from dengue virus serotype 1 (DENV-1) to DENV-2 in January 2007 and cocirculation of all 4 serotypes with general dominance of DENV-1 and DENV-2 and lesser circulation of DENV-3 and DENV-4. *From ≈10% of all dengue cases.

Early detection of this switch warned of a possible upcoming dengue outbreak. In response, an enhanced vector-control program was activated in February 2007. The proportion of DENV-2–positive samples detected by PCR rose from 57.9% in January 2007 to a peak of 91.0% in July 2007. This increase was accompanied by an increase in the total number of dengue cases reported by the Ministry of Health; cases peaked at 432 in the first week of July 2007. By late August, the number of dengue cases fell to below the warning level (warning level = 256 cases/epidemiologic week) as reported by the Ministry of Health (*9*). During the switch in predominant serotype, fatality rates (0.32% in 2006 and 0.27% in 2007) and dengue hemorrhagic fever rates (2.4% in 2006 and 2.1% in 2007) did not differ substantially among the reported cases. During this same period of extensive surveillance, 5.2% of the samples in 2007 and 10.8% in 2008 were detected as DENV-3. Our spatial analysis indicated localized emergence of DENV-3 in the eastern region of the country in 2007 and in the central region in 2008. Enhanced control was also attempted in these areas to prevent the spread of the serotype that had been uncommon in Singapore.

Phylogenetic analysis of DENV-2 envelope gene sequences showed that the switch in predominant serotype in early 2007 coincided with a clade replacement within DENV-2. During 2000–2008, 2 distinct subclades, with strong temporal topology, were found within the cosmopolitan genotype (Figure 2). Specifically, DENV-2 isolates obtained before 2007 formed the subclade herein referred to as the old clade, whereas isolates obtained in 2007 and later formed the new clade with strong bootstrap support. Because 1 of the DENV-2 isolates sampled in 2005 clustered with the new clade but fell closer to the root of that clade, in situ evolution giving rise to DENV-2 viruses that subsequently replaced the old clade viruses is highly likely. A GenBank sequence that belonged to DENV-2, sampled in 2007 in Vietnam, grouped within the new clade, indicating that this virus strain was not re-

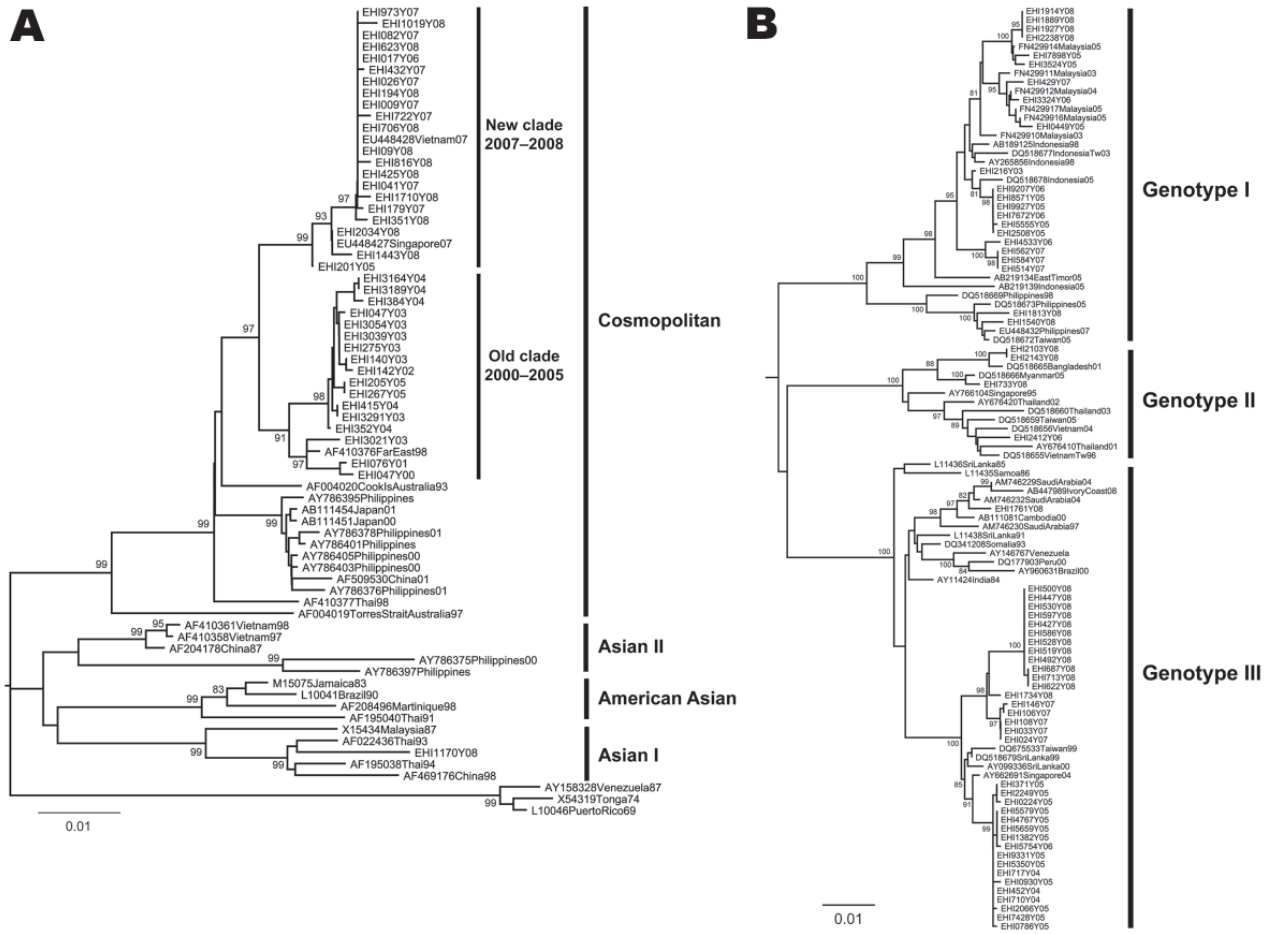

Figure 2. Maximum-likelihood tree showing the phylogenetic relationship of A) dengue virus serotype 2 (DENV-2) and B) DENV-3 from Singapore and global isolates based on the envelope protein gene. EHI, sequence data generated at Environmental Health Institute; new clade, isolates obtained in 2007 and later; old clade, isolates obtained before 2007. Numbers on branches represent bootstrap percentages; only those >80% are shown. Scale bars indicate substitutions per site.

stricted to Singapore and may have been circulating in this region.

Our dengue surveillance also indicated sporadic emergence of DENV-3 from localized areas throughout the country (*6*,*10*). Phylogenetic analysis of isolates from Singapore from 2006 through 2008 identified 3 genotypes of DENV-3. These isolates were closely related to those found in Indonesia, Malaysia, Philippines, Thailand, Saudi Arabia, and Côte d'Ivoire (Figure 2), which suggests multiple importations of DENV-3 viruses into Singapore. Analysis of DENV-1 sequences showed that all except 3 belonged to genotype I and were similar to those responsible for the 2005 outbreak (data not shown).

Conclusions

Our dengue surveillance provided early warning of the outbreak in 2007 and contributed to early activation of enhanced vector control. Although we were unable to assess the effectiveness of the control measures, considering the regional situation in 2007 (*11*,*12*), we believe that without these measures the dengue situation in Singapore in 2007 would have been worse than or comparable to that in 2004–2005. After a lull year in 2006, dengue cases were expected to rise for a few years. The integrated vector control program has interrupted the dengue trend, with 7,032 cases reported in 2008 and 4,498 in 2009.

As a travel hub, Singapore experiences continuous importation of dengue viruses. Although some become established at various levels, some develop into outbreaks and subsequently get replaced. Our study demonstrates how rapidly dengue virus serotypes can be replaced within a population. It also highlights the complexity of the disease and the challenges faced by affected states that seek to understand the epidemiology for purposes of disease control. To shed further light on the complex interplay among the various factors that affect dengue transmission, studies are being conducted on complete genome sequences of dengue viruses, vectorial capacity of local *Aedes* spp. mosquitoes, and cross-reactive immune responses to different dengue serotypes.

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Adenovirus 36 DNA in Adipose Tissue of Patient with Unusual Visceral Obesity

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Massive adipose tissue depositions in the abdomen and thorax sufficient to interfere with respiration developed in a patient with multiple medical problems. Biopsy of adipose tissue identified human adenovirus 36 (Adv 36) DNA. Adv 36 causes adipogenesis in animals and humans. Development of massive lipomatosis may be caused by Adv 36.

Infection with human adenovirus 36 (Adv 36) has been reported to cause a large accumulation of fat in 4 animals (chickens, mice, rats, and monkeys) (*1–3*). Selective deposition of visceral fat disproportional to total fat deposition was observed in some studies. The increase in visceral fat or total body fat in infected animals compared with uninfected animals was >100% in some experiments (*1–3*). Of animals that were infected, 60%–100% became obese compared with uninfected animals (*1–4*). Obesity was defined as a weight or fat content greater than the 85th percentile of the uninfected animals.

Several human studies have shown a correlation of antibodies to Adv 36 and obesity (*4–8*). In 1 study of >500 persons, 30% of obese persons and 11% of lean persons had antibodies to Adv 36 (*4*). The body weight of infected persons was ≈25 kg heavier than that of uninfected persons (*4*). In 26 pairs of twins with discordant Adv 36 antibody status, infected twins were heavier and fatter (*4*). In a group of obese school children from South Korea, 30% had antibodies to Adv 36, and infected children had higher body mass index z-scores than uninfected children (*5*). However, in animals and adults in the United States, serum cholesterol and triglyceride levels were paradoxically reduced, despite the obesity (*1–4*). Recent reports of adults in Italy and children in South Korea support the association of Adv 36 and obesity, and show that Adv 36 is more common in obese persons; prevalence ranges from 29% to 65% (*6,7*).

The mechanisms responsible for the increased adiposity are changes in gene expression of multiple enzymes and transcription factors by the virus (*8–15*). In adipocytes, the sterol regulatory element binding protein pathway is increased, resulting in increases in levels of sterol regulatory element binding protein 1 and fatty acid synthase. Because levels of transcription factor CCAAT/enhancer binding protein-β, peroxisome proliferator-activated receptor-γ, and lipoprotein lipase are also increased, lipid transport into cells and fatty acid synthesis within cells is increased (*8–15*). In muscle cells, gene expression of glucose transporters Glut 1 and Glut 4 and phosphoinositide 3-kinase is increased, which results in noninsulin-mediated increases in glucose transport (*14*).

These changes are thought to be caused by the action of the Adv 36 open reading frame 1 early region 4 gene and may be blocked by small interfering RNA or the antiviral drug cidofovir (*11,13*). When the open reading frame 1 early region 4 gene was transferred to a retrovirus and inserted into preadipocytes in vitro, the gene was capable of inducing the enzymes and enhancing fat accumulation (*13*).

Adv 36 DNA persists in multiple tissues of infected animals for long periods after initial infection (*3*). Viral DNA was isolated from brain, lung, liver, muscle, and adipose tissue of monkeys 7 months after initial infection, long after the active virus has disappeared from blood and feces (*3*). The virus DNA apparently continues to alter gene expression chronically in tissues.

We report a patient with massive fat deposits in the thorax and abdomen. We postulate that these abnormal adipose tissue deposits were caused by Adv 36 infection.

The Patient

The patient, a 62-year old man who was diagnosed with high-grade large cell lymphoma in 1999, received multidrug (cyclophosphamide, doxorubicin, vincristine, and prednisolone) chemotherapy, central nervous system prophylaxis with cytarabine, and high-dose methotrexate. In February 2000, he underwent autologous bone marrow transplantation and received a conditioning regimen of etoposide, cytoxan, and fractionated total-body irradiation. Hypothyroidism, chemoradiation-induced hypogonadism, and adrenal insufficiency developed in the patient, which required chronic glucocorticoid replacement.

During the next 7 years, prostate cancer, rectal ulcer necessitating colon diversion, hemolytic anemia, thrombocytopenia, myelodysplastic syndrome, and diabetes mellitus developed in the patient; he was treated with insulin for the diabetes. He was hospitalized for respiratory insufficiency in August, 2007, which was thought to be caused or exacerbated by massive intrathoracic and intraabdomi-

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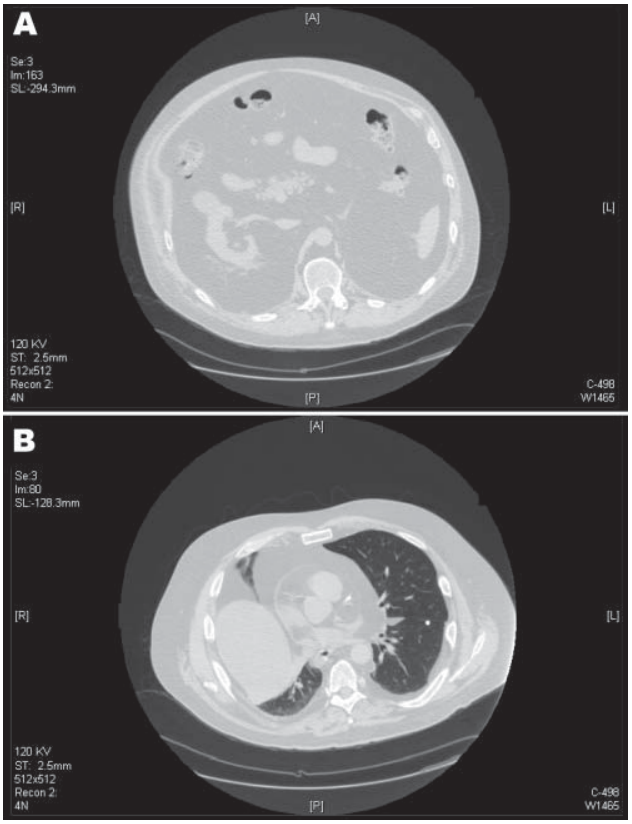

Figure 1. Computed tomography scans of the patient, showing marked visceral adipose tissue in the abdomen (A) and thorax (B). Diffuse intrabadominal, retroperitoneal lipomatosis, and herniation of the mediastinum can be seen through the esophageal hiatus. Intrapericardial adipose infiltration and adipose tissue bilaterally are seen within the pleura.

nal fat deposits. He had obesity of his neck, lateral chest, and abdomen, but limited subcutaneous fat in his abdomen and upper extremities. He had no buffalo hump, round facies, or other signs of Cushing syndrome. The patient had tachycardia with muffled heart sounds, dullness in the base of the right chest, and bibasilar diminished breath sounds. A computed tomography scan of the chest and abdomen showed fatty densities extending into the intrabdominal, intraperitoneal, and retroperitoneal areas and herniating through the esophageal hiatus into the mediastinum (Figure 1). These fatty densities extended within the pericardium without definite pericardial effusion.

The patient's weight was 113 kg, height 183 cm, body mass index 34, waist circumference 145 cm, and hip circumference 111 cm. Laboratory tests showed triglycerides 1.356 mmol/L, total cholesterol 2.2015 mmol/L, high-density lipoprotein cholesterol 0.5957 mmol/L, and low-density lipoprotein cholesterol 0.9842 mmol/L. The serum lipids values represent marked decreases from previous measurements. In December 2002, his low-density lipoprotein cholesterol level was 2.7412 mmol/L. In April 2007, his serum triglyceride level was 4.92244 mmol/L. The result of a test for serum immunoglobulin (Ig) M against adenoviruses was negative (0.07 IU), and the result of a test for serum IgG was positive (2.18 IU).

Infection with Adv 36 causing disseminated lipomatosis was suspected. A subcutaneous fat biopsy specimen was assayed for Adv 36 DNA by nested PCR (*4*). Three of 4 adipose tissue samples showed a band compatible with Adv 36 DNA. Water controls in the assay had negative results. A *Hae*III digest of the presumed Adv 36 DNA band showed digestion at the expected site and yielded 2 bands of equal size (Figure 2). Sequencing of the DNA band by the Virginia Commonwealth University Massey Cancer Center Molecular Biology Core (Richmond, VA, USA) identified the sequence as Adv 36 DNA.

As a control, samples of adipose tissue obtained by needle fat biopsy from 12 obese persons without abnormal adipose tissue deposits were evaluated by nested PCR and quantitative PCR by using proprietary Taqman primers and probe (Obetech, Richmond, VA, USA). These persons provided written informed consent. Samples for quantitative PCR were analyzed with an ABI Step One PCR apparatus (Applied Biosystems, Foster City, CA, USA). Two of the 8 samples assayed by nested PCR were positive and 5 of 12 samples assayed by quantitative PCR were positive. The prevalence of Adv 36 infection identified by PCR was similar to that identified by serum neutralization in obese adults in the United States (*5*).

Conclusions

Adv 36 DNA in the adipose tissue of this patient documents that he was infected with this virus. The propensity

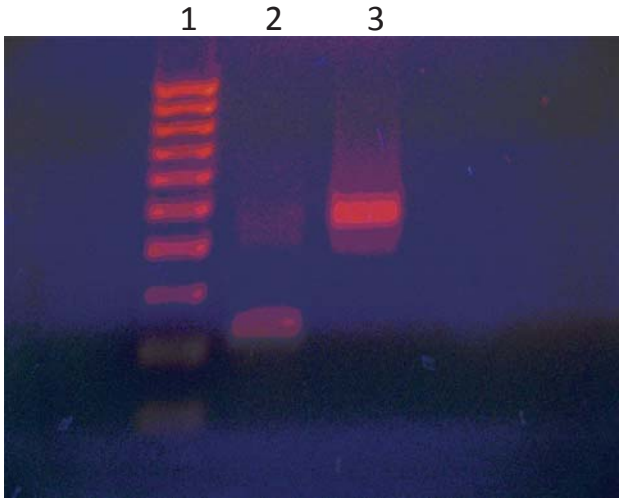

Figure 2. *Hae*III digestion of adenovirus 36 (Adv 36) DNA PCR products of the patient. Lane 1, molecular size marker; lane 2, *Hae*III digest of Adv 36 DNA; lane 3, undigested Adv 36 DNA.

of Adv 36 to increase visceral adipose tissue in experimentally infected animals suggests that the abnormal adipose tissue deposits within the abdomen and chest cavities and in the subcutaneous spaces of the chest and neck could be caused by Adv 36 infection. He was being treated with replacement corticosteroids but did not have signs of Cushing syndrome.

More research is needed to determine if Adv 36 plays a role in abnormal adipose tissue deposits/lipomatosis. If Adv 36 is found to be a cause, research is needed to identify effective antiviral agents with a more tolerable side effect profile. Cidofovir is effective against Adv 36 in vitro, but has major side effects in humans.

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Vitamin D Deficiency and Tuberculosis Progression

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To assess the association between vitamin D deficiency and tuberculosis disease progression, we studied vitamin D levels in a cohort of tuberculosis patients and their contacts (N = 129) in Pakistan. Most (79%) persons showed deficiency. Low vitamin D levels were associated with a 5-fold increased risk for progression to tuberculosis.

Deficiency of vitamin D (25-hydroxycholecalciferol) has long been implicated in activation of tuberculosis (TB) (*1*). Serum levels of vitamin D in TB patients are lower than in healthy controls (*2*,*3*). Paradoxically, prolonged treatment of TB also causes a decline in serum vitamin D levels (*2*). Several studies have suggested that vitamin D is a potent immunomodulator of innate immune responses (*4*,*5*) by acting as a cofactor for induction of antimycobacterial activity (*6*). Of the 22 countries that have the highest TB incidence, Pakistan ranks eighth. In a previous study in Karachi, we observed that active disease developed in 7 (6.4%) of 109 TB case-contacts within 2 years (*7*). In the present study, we explored the role of vitamin D deficiency in TB disease progression within this cohort.

The Study

Household contacts (n = 109) of 20 patients with recently diagnosed sputum-positive pulmonary TB (index case-patients) were enrolled at Masoomeen General Hospital, in Karachi during 2001–2004 for a TB household cohort study (*7*). Blood samples were collected at baseline and at 6, 12, and 24 months follow-up. Visiting health workers reviewed clinical charts every 3 months for the first 24 months and at a final home study visit during November 2007–January 2008 (45–74 months from baseline). Persons with secondary cases were referred to a consultant at Masoomeen General Hospital for additional investigation, including assessment of physical signs and symptoms, laboratory tests, chest radiographs, and sputum smear microscopy (*7*). For the present study, 129 de-identified, plasma samples preserved at –70°C from the baseline visit were shipped to Stanford University (Stanford, CA, USA) for analysis of vitamin D levels. Total circulating serum 25[OH] vitamin D was measured with ELISA by using the Immuno Diagnostic System Ltd (IDS, Fountain Hill, AZ, USA). All protocols were followed according to manufacturer's instructions. Each test was run in duplicate, with mean absorbance computed from the average for 2 wells normalized to a zero calibrator well. Levels of vitamin D in test samples were derived by fitting a 2-parameter logistic curve to 6 standard levels and expressed as ng/mL (1 nmol/L × 0.4 = 1 ng/mL). All R^2 values were >95%. The assay detection range was 6–360 nmol/L (2.4–144 ng/mL). Levels in 1 person were below the detection limit and were excluded from analysis. The ethical review committees of Aga Khan and Stanford universities approved the study protocol.

We used Kaplan-Meier analysis to evaluate the association of vitamin D levels with outcome of TB disease in 100 household contacts completing ≥1 follow-up visit. Vitamin D levels in the cohort were classified in population-based tertiles (low, middle, high). We used SAS version 9.3 (SAS Institute, Cary, NC, USA) for statistical analyses.

Median vitamin D level for the 128 cohort participants was 9.1 ng/mL (interquatrile range [IQR] 5.3–14.7); levels were 9.6 ng/mL (IQR 5.8–19.1) for 100 disease-free contacts, 7.9 ng/mL (IQR 4.7–10.3) for 20 TB index case-patients, 4.6 ng/mL (IQR 4.0–5.2) for 2 co-prevalent TB case-patients who were receiving antituberculous treatment at recruitment, and 5.1 ng/mL (IQR 3.4–14.3) in 6 household contacts with a history of TB treatment (2–10 years) (Figure 1, panel A). In the 100 disease-free household contacts, vitamin D levels were significantly higher than in the 28 participants with a history of TB diagnosis at baseline (p = 0.02; Mann-Whitney U test) (Figure 1, panel B). Median vitamin D levels were significantly lower in the 74 female patients than in the 54 male patients (7.8 vs. 11.9, Mann-Whitney U test, p = 0.0004) (Figure 1, panel C). When we stratified the cohort by vitamin D level, 79% had deficient (<20 ng/mL), 14% had insufficient (20–30 ng/mL), and 7% had sufficient (>30 ng/mL) levels of vitamin D (Table).

We next analyzed risk for progression to active TB in relation to plasma vitamin D levels. Of the 100 disease-free household contacts, 8 (8%) progressed to active disease during 4 years of follow-up. TB progression was significantly associated with relatively lower plasma vitamin D levels (Figure 2). Disease progressed in 7 (23%) of 30 patients with plasma vitamin D levels in the lowest tertile (<7 ng/mL), 1 (3%) of 32 with vitamin D levels in the middle tertile (7–13 ng/mL), and none of 30 in the highest tertile (>13 ng/mL) (p = 0.002, log rank). Six (75%) of 8 patients whose TB progressed were female patients with vitamin D

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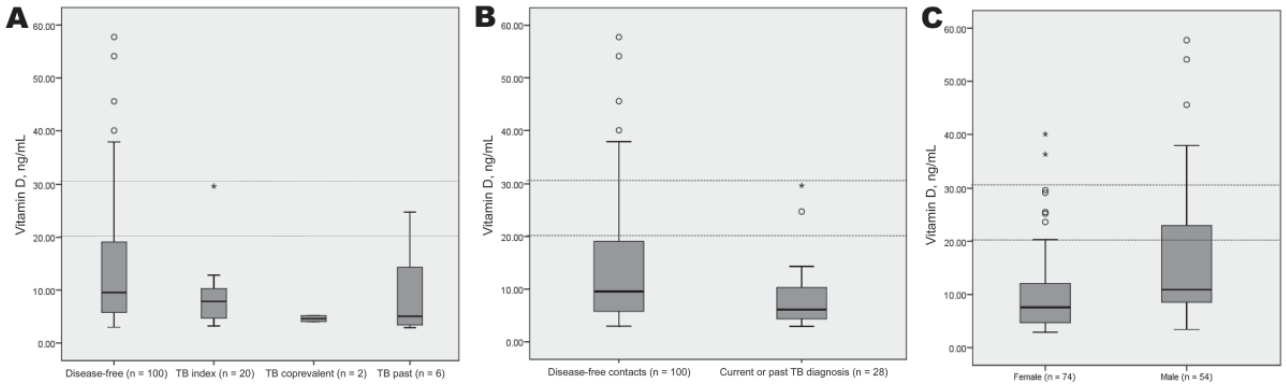

Figure 1. Levels of vitamin D in plasma in the Karachi, Pakistan, tuberculosis (TB) household cohort (*7*) by TB status at baseline (disease-free, index TB case-patient, coprevalent TB case-patient, and past TB case-patient, treated 2–10 years previously). One disease-free contact was excluded because of an indeterminate test result. Box plots show the median, 25th, and 75th quartiles of serum vitamin D estimated for each group (A) by any TB diagnosis (current or past) at baseline (B) and by sex (C). Reference lines represent cut-offs for insufficient and sufficient vitamin D levels, respectively. The Mann-Whitney U test was used for comparison of medians.

levels in the lowest tertile. Further adjustment for age and sex yielded a relative risk for progression of 5.1 (1.2–21.3, p = 0.03) for a relative 1-log decrement in vitamin D levels, which suggests that vitamin D deficiency might be a strong risk factor for TB disease.

Conclusion

In this cohort follow-up study from Pakistan, low vitamin D levels were associated with progression to active TB disease in healthy household contacts. No deaths occurred during the follow-up period from either TB or unrelated causes. Our findings also suggest that vitamin D deficiency may explain the higher susceptibility of women to disease progression in our cohort. A high prevalence of vitamin D deficiency in female patients also was reported in ambulatory patients at Aga Khan University (*8*). Factors such as low socioeconomic status, poor nutrition, traditional/cultural traits, and little exposure to sunlight may further explain vitamin D deficiency in female patients in this cohort. Despite several limitations to our study, such as information about diet, body mass index, exposure to sunlight and the relatively small number of study participants, our results are supported by a meta-analysis of 7 case-control studies in different ethnic populations (including an Indian population) that showed 70% of healthy controls had higher vitamin D levels than did untreated TB patients (*3*). Previously in African immigrants in Melbourne, Victoria, Australia (*9*), lower mean vitamin D levels were associated with high probability of latent, current, or past TB infection. Cross-sectional studies are needed in Pakistan to appreciate this association with sex and susceptibility to TB with larger sample size. Most of the South Asian population, including Pakistani immigrants to European countries and South Indians, had <10 ng/mL of serum vitamin D level (*10*) and is consistent with reports from Aga Khan Hospital (*8*,*11*). Vitamin D plays an important role in activation of 1 α-hydroxylase to convert 25(OH) D to its active form [1, 25 (OH) 2D] that leads to expression of cathelicidin, a microbicidal peptide for *Mycobacterium tuberculosis* (*5*,*12*). Serum levels >30 ng/mL provide an adequate substrate for

Table. Prevalence of vitamin D deficiency in a cohort study of household contacts of tuberculosis patients, Karachi, Pakistan*

Baseline characteristic	Total, no. (%)	Deficient, no. (%)	Insufficient, no. (%)	Sufficient, no. (%)	p value
Age group, y					
6–17	44 (34)	38 (86)	4 (9)	2 (5)	0.13
≥18	84 (66)	63 (75)	14 (17)	7 (8)	
Sex					
Male	54 (42)	35 (65)	12 (22)	7 (13)	0.003
Female	74 (58)	66 (89)	6 (8)	2 (3)	
Tuberculin skin test					
≥10 mm	101 (79)	81 (80)	14 (14)	6 (6)	0.49
<10 mm	27 (21)	20 (74)	4 (15)	3 (11)	
Total	128 (100)†	101 (79)	18 (14)	9 (7)	

*Deficient, <20 ng/mL; insufficient, 20–30 ng/mL; sufficient, >30 ng/mL. p values derived by χ^2 test for comparison of deficient and insufficient or sufficient levels.
†Excluding 1 person with indeterminate result.

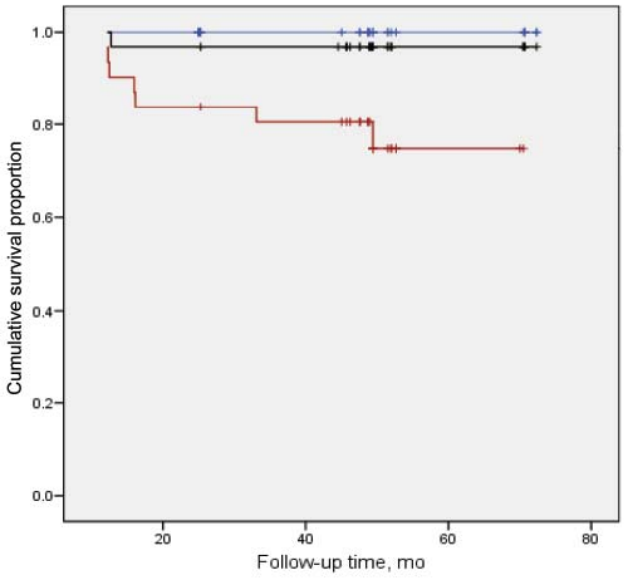

Figure 2. Risk for tuberculosis (TB) progression, by baseline plasma vitamin D level. Risk for progression in 100 household contacts of TB patients are indicated in cohort-based tertiles of vitamin D levels in plasma at baseline: lowest, <7.4 ng/mL (red); middle, 7.4–13 ng/mL (black); highest, >13 ng/mL (blue). Plus signs indicate censoring points. Events are defined as time to diagnosis of active TB disease during follow-up.

the enzyme. Serum levels <20 ng/mL may therefore impair the macrophage-initiated innate immune response to *M. tuberculosis* and offer a possible explanation for geographic and ethnic (*13*) variations in susceptibility to TB.

Vitamin D supplementation during TB treatment remains controversial; a few studies have reported clinical improvement in pulmonary TB (*14*) and 1 study reported no effect (*15*). However, our findings indicate that further studies should be conducted regarding use of vitamin D as a supplement for persons undergoing treatment for TB and those with latent TB infection.

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La Crosse Virus in *Aedes albopictus* Mosquitoes, Texas, USA, 2009

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We report the arthropod-borne pediatric encephalitic agent La Crosse virus in *Aedes albopictus* mosquitoes collected in Dallas County, Texas, USA, in August 2009. The presence of this virus in an invasive vector species within a region that lies outside the virus's historically recognized geographic range is of public health concern.

La Crosse virus (LACV) is the most common cause of arthropod-borne, pediatric encephalitis in North America. A member of the California serogroup within the family *Bunyaviridae* and the genus *Orthobunyavirus,* LACV is enveloped and contains a negative-sense, tripartite genome with segments designated small (S), medium (M), and large (L). Cases of LACV-associated encephalitis, which can be fatal, occur within the geographic range of its principal vector, *Aedes triseriatus* mosquitoes. This native tree-hole breeding mosquito is distributed throughout wooded regions east of the Rocky Mountains within the United States. Historically, most LACV-associated encephalitis cases have occurred in upper midwestern states, including Wisconsin, Illinois, Minnesota, Indiana, and Ohio (Figure 1). In recent years, LACV encephalitis activity has increased above endemic levels in regions of the southeastern United States, including West Virginia, North Carolina, and Tennessee (Figure 1) (*1*). In addition, recent cases of LACV encephalitis have been reported as far south as Louisiana, Alabama, Georgia, and Florida (Figure 1).

Ae. albopictus is an invasive mosquito species that was first discovered in Houston, Texas, in 1985 (*2*); having apparently arrived in the United States in a shipment of used tires from Asia (*3*). An opportunistic container-breeder, its vector competence for many arthropod-borne viruses (arboviruses), including LACV, and its catholic feeding habit have made the invasion of *Ae. albopictus* mosquitoes disconcerting to researchers, who have warned of the potential for an increased incidence of vector-borne diseases as a result (*4*,*5*). Since 1985, the geographic distribution of these mosquitoes has grown to include most of the southeastern United States. The concurrent increase in LACV encephalitis activity has led to speculation on the possible transmission of LACV by *Ae. albopictus* mosquitoes as an accessory mechanism to the historically recognized transmission by *Ae. triseriatus* mosquitoes (*6*). LACV has been isolated from *Ae. albopictus* mosquitoes in Tennessee and North Carolina in 1999 and 2000, respectively, during a period of greatly increased LACV activity in those areas (*6*). However, the role of this species in LACV transmission remains unknown.

We report the isolation of LACV from a pool of 3 *Ae. albopictus* mosquitoes collected outside the known geographic range of the virus, in Dallas County, Texas, on August 13, 2009 (Figure 1). This is one of only several isolations of LACV within the state; the first isolate was derived from a pool of *Ae. infirmatus* mosquitoes collected in Houston in 1970 (*7*). After the identification of LACV in the Dallas pool, an additional isolation of LACV was made from a mixed pool of 29 *Ae. albopictus* and 2 *Ae. triseriatus* mosquitoes collected in Fort Bend County, Texas, in October 2009 (Figure 1). The Fort Bend County location is relatively near the site of collection of the 1970 Texas

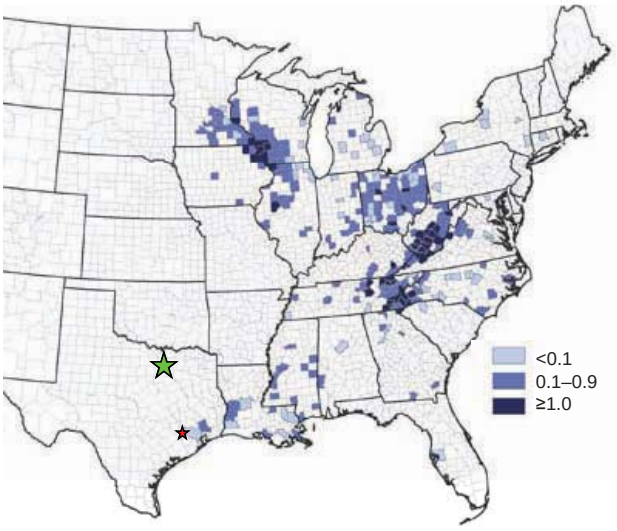

Figure 1. Geographic distribution of La Crosse virus (LACV) in accordance with the habitat range of *Aedes triseriatus* mosquitoes in the United States as inferred from the California serogroup virus neuroinvasive disease average annual incidence by county, 1996–2008. Incidence rates are shown in shades of blue. Dallas County and Fort Bend County locations of the 2009 LACV isolations from pools containing *Ae.albopictus* and *Ae. triseriatus* mosquitoes are indicated by green and red stars, respectively. Data and figure adapted from the Centers for Disease Control and Prevention website (www.cdc.gov/lac/tech/epi.html).

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LACV-positive pool and the known geographic distribution of LACV activity in southeastern Texas and Louisiana (Figure 1). Taken together, our results represent an unprecedented number of LACV findings within the state of Texas.

The Study

As part of ongoing arbovirus surveillance efforts, the City of Dallas Vector Control Division collected 65 mosquitoes in a gravid trap at the edge of a wooded area near a residential district in Dallas County on August 13, 2009. Upon their receipt at the Texas State Department of Health Services, none of the mosquitoes was viable. The mosquitoes were sorted and identified by sex. Female mosquitoes were grouped into 3 pools by species: pool no. AR6318, consisting of 50 *Culex quinquefasciatus* mosquitoes, pool no. AR6319, consisting of 3 *Ae. albopictus* mosquitoes; and pool no. AR6320, consisting of 1 *Ae. triseriatus* mosquito.

Generated pools were macerated in 1.5 mL of bovine albumin diluent arbovirus medium followed by 2 rounds of centrifugation at 10,000 rpm for 5 min each. Between each round of centrifugation, a rest period of 15 min was used to facilitate pellet formation. After centrifugation, 50 μL of the resultant supernatant was injected onto BHK and Vero cells. These cells were incubated at 37°C and examined for cytopathic effect (CPE) over the next 10 days. At day 5 postinoculation, Vero cells inoculated with the supernatant derived from pool no. AR6319 (*Ae. albopictus*) demonstrated marked CPE. This condition represented a preliminary virus isolation-positive result. No CPE was observed in the BHK cells. Infected cells were then subjected to immunofluorescent antibody assays with antibodies directed against various arboviruses, followed by the use of fluorescein isothiocyanate–conjugated antimouse antibodies for detection. From these analyses, the isolate derived from pool no. AR6319 (*Ae. albopictus*) was determined to be a California serogroup virus. Furthermore, pool no. 6318 (*Cx. quinquefasciatus*) tested positive for West Nile virus, and pool no. 6320 (*Ae. triseriatus*) was negative for virus by the above described methods.

To further identify the California serogroup virus identified in pool no. AR6319 (*Ae. albopictus*), the pool and the Vero cell–derived isolate were sent to the Centers for Disease Control and Prevention in Fort Collins, CO, USA, for additional testing. Upon receipt of the samples in Fort Collins, a reverse transcription–PCR was performed to amplify cDNAs from all 3 segments of the orthobunyavirus genome by using the consensus oligonucleotide primers shown in the Table and conditions and methods previously described (*8*). Generated cDNAs were then subjected to nucleotide sequencing and BLAST (www.ncbi.nlm.nih.gov/BLAST) analyses; the results indicated that the pool and the isolate were positive for LACV S, M, and L segment RNAs.

Subsequently, a pool (AR8973) of 29 *Ae. albopictus* and 2 *Ae. triseriatus* mosquitoes collected in Fort Bend County, Texas on October 5, 2009, was identified as positive for LACV S, M, and L segment RNAs by using the same processing and characterization methods described above. After these analyses, full-length S, M, and L segment genomic sequences (GenBank accession nos. GU591164–9) were generated for LACV RNAs extracted from LACV-positive pools and Vero cell isolates by using oligonucleotide primers specific for the previously published LACV prototype genome (human 1960, GenBank accession nos. EF485030–2) and methods previously described (*9*).

Phylogenetic analyses of partial LACV M segment sequences (Figure 2) indicate that the LACVs present in the Texas 2009 pools are closely related to LACVs isolated from Alabama, Georgia, and New York of the previously described lineage 2 (*11*) and genotype C (*7*) designations. These findings suggest a likely southeastern ancestry for the Texas 2009 LACV isolates.

Conclusions

The presence of LACV in *Ae. albopictus* mosquitoes in Dallas County, Texas, in late summer 2009 represents the possible expansion of the geographic range of an endemic pathogen within this invasive mosquito species in the United States. The subsequent occurrence of LACV in Fort Bend County in October 2009 should be of concern to public health practitioners who have been alerted to the

Table. Orthobunyavirus consensus oligonucleotide primers used for amplification and sequencing of La Crosse virus partial S, M, and L segment cDNAs, Texas, 2009*

Targeted genomic regions	Name	Primer sequence (5′ → 3′)	Approximate amplicon size, bp
S segment nucleocapsid ORF	Cal S forward	GCAAATGGATTTGATCCTGATGCAG	210
	Cal S reverse	TTGTTCCTGTTTGCTGGAAAATGAT	
M segment 5′ terminus/glycoprotein ORF	Ortho M 5′ terminus	AGTAGTGTACTACC	410
	Ortho M ORF reverse	TTRAARCADGCATGGAA	
L segment 5′ terminus/polymerase ORF	Ortho L 5′ terminus	AGTAGTGTACTCCTA	550
	Ortho L ORF reverse	AATTCYTCATCATCA	

*Oligonucleotide primers designed against conserved regions of the orthobunyavirus genome. S segment primers appear in a previous publication (*8*). All primers were applied in singleplex reactions using methods described previously (*8*) with altered primer annealing conditions of 50°C for 1 min. S, small; M, medium; L, large; ORF, open reading frame.

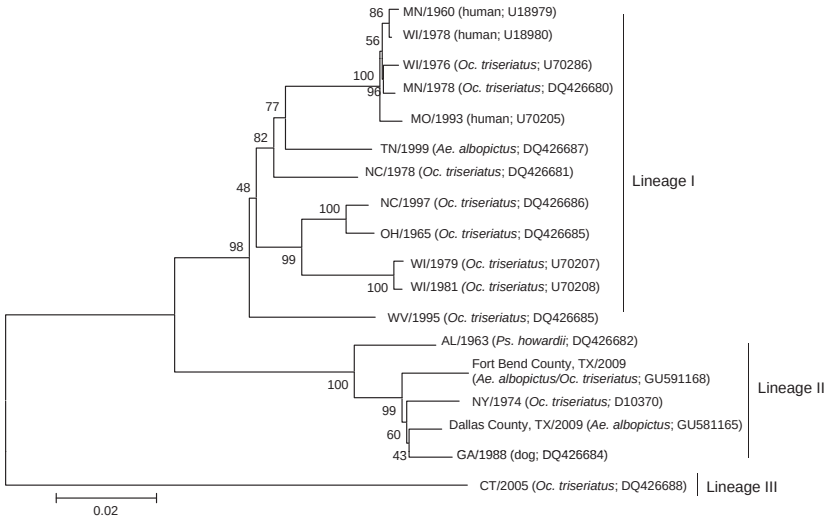

Figure 2. Phylogeny of La Crosse virus (LACV) medium (M) segment sequences of diverse origins. According to a limited availability of full-length sequences in GenBank, 1,663 nt of the M segment glycoprotein gene open-reading frame are compared. Isolate source and GenBank accession nos. appear after the isolate designation for each taxon. Sequences were aligned by ClustalW (*10*) and neighbor-joining and maximum-parsimony trees were generated by using 2,000 bootstrap replicates with MEGA version 4 software (*10*). Highly similar topologies and confidence values were derived by all methods and a neighbor-joining tree is shown. Scale bar represents the number of nucleotide substitutions per site. The 2009 Texas (TX) isolates group with strong support with lineage 2 viruses of the extreme south and New York (NY), which suggests a likely southern origin for LACV isolates. MN, Minnesota; WI, Wisconsin; *Oc., Ochlerotatus*; MO, Missouri; TN, Tennessee; *Ae., Aedes*; NC, North Carolina; OH, Ohio; WV, West Virginia; AL, Alabama; *Ps., psorophora*; GA, Georgia; CT, Connecticut.

presence of this pathogen near 2 major urban centers, Dallas and Houston. Of interest, San Angelo virus, which is serologically related to LACV, is known to occur in Texas and has been shown to replicate in and be transovarially transmitted by *Ae. albopictus* mosquitoes (*12*), although this virus has no known association with human disease. Cocirculation enables possible reassortment of genomic segments between LACV and San Angelo virus, a phenomenon that has been described for viruses of the California serogroup within *Ae. albopictus* mosquitoes (*13*) with unknown public health outcomes.

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Unusual Assortment of Segments in 2 Rare Human Rotavirus Genomes

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Using full-length genome sequence analysis, we investigated 2 rare G3P[9] human rotavirus strains isolated from children with diarrhea. The genomes were recognized as assortments of genes closely related to rotaviruses originating from cats, ruminants, and humans. Results suggest multiple transmissions of genes from animal to human strains of rotaviruses.

Group A rotaviruses possess a genome of 11 segments of double-stranded RNA (*1*). Rotaviruses are associated with acute gastroenteritis in humans and a wide variety of other mammalian and avian species (*1*). The evolution and diversity of rotaviruses is driven by genomic reassortment, accumulation of point mutations, intragenic recombination, and interspecies transmission (*2*,*3*). At least 23 G genotypes (structural viral protein [VP] 7 related) and 32 P genotypes (VP4 related) have been identified thus far in rotaviruses (*4*). Unlike other G and P types, G3 has been identified in rotavirus strains from humans and from almost all other susceptible mammalian species, including dogs, cats, monkeys, horses, rabbits, pigs, and ruminants, in association with various P types, thus exhibiting a broad host range (*1*). G3 human rotaviruses are usually associated with P[8] or P[6] and, rarely, with P[9] (5,*6*).

Historically, RNA–RNA hybridization has been used to study the genetic relationships among rotavirus strains and has shown 2 major pools among human rotaviruses, named Wa-like and DS-1–like (*7*). Recently, a new classification system based on whole-genome sequence analysis enabled researchers to better understand the complex interactions between human and animal rotaviruses (*8*,*9*). Application of this new classification system showed a close evolutionary relationship between human Wa-like and porcine rotavirus strains and between human DS-1–like and bovine rotavirus strains, suggesting that the 2 major human rotavirus G and P types might have an animal origin (*8*). A third human rotavirus family, designated AU-1–like, comprises a group of globally circulating but overall rare strains, mainly with the G3P[9] combination. Early RNA-RNA hybridization studies suggested a genetic relationship of particular human G3P[9] strains with feline rotaviruses (*10*). Later, feline–bovine reassortant G3P[9] rotaviruses were also identified in humans (*11*). However, because of the limits of resolution of the RNA–RNA hybridization method, determining the exact origin of individual genome segments in these strains was not possible.

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The Study

During uninterrupted surveillance for human rotaviruses in Palermo, Italy, which started in the mid-1980s, 3 strains (PAF96/94, PAH136/96, and PAI58/96) were detected that displayed AU-1–like features because they possessed long electropherotype, subgroup (SG) I (VP6 related) and G3P[9] genotypes. The viruses were identified from children <5 years of age who were hospitalized with acute gastroenteritis at the "G. Di Cristina" Children's Hospital of Palermo in 1994 and 1996. Sequence analysis found all 3 strains to be genetically related to strains of either human or feline origin in the VP7, VP4, and VP6 genes. In contrast, the nonstructural protein (NSP) 4 gene of these viruses resembled that of G2P[4] human strains, suggesting a reassortment between AU-1–like and DS-1–like strains (*5*). To understand the evolution and origin of these viruses, we determined the full-length genome sequence of 2 such unusual G3P[9] viruses, strain PAH136/96 and PAI58/96, that appeared to be genetically distinct and for which enough material was available for additional analyses. The complete genome sequences were obtained as described elsewhere (*12*). Genome sequences were individually compared with cognate sequences of a variety of rotavirus strains by phylogenetic analysis by using MEGA4 software (www.megasoftware.net). In addition, the sequences were analyzed by using BLAST (www.ncbi.nlm.nih.gov/BLAST) with default search values. The GenBank nucleotide sequence accession numbers of PAH136/96 and PAI58/96, respectively, are GU296430 and GU296431 for VP7, GU296426 and GU296427 for VP4, GU296428 and GU296429 for VP6, GU296420 and GU296421 for VP1, GU296422 and GU296423 for VP2, GU296424 and GU296425 for VP3, GU296410 and GU296411 for NSP1, GU296412 and GU296413 for NSP2, GU296414 and GU296415 for NSP3, GU296416 and GU296417 for NSP4, GU296418 and GU296419 for NSP5.

The genomes of the PAH136/96 and PAI58/96 G3P[9] strains were 18,485 nt long. The 2 strains from Italy pos-

Table 1. Nucleotide identity of 11 genome segments of 2 human rotavirus strains, Italy, 1994 and 1996*

Gene encoding	Cutoff value	Genotype of PAH136/96	Identity of PAH136/96 against indicated strains		Genotype of PAI58/96	Identity of PAI58/96 against indicated strains		Identity between PAH136/96 and PAI58/96
			Prototypes†	GenBank strains‡		Prototypes†	GenBank strains‡	
VP1	83	R2	83.7 (RF)	94.6 (Hun5)	R2	94.9 (RF)	95.7 (NCDV)	83.2
VP2	84	C2	90.9 (RF)	98 (Hun5)	C2	89.9 (RF)	93.9 (Chubut)	91.6
VP3	81	M2	89.2 (RF)	92.9 (PA169)	M2	90.2 (RF)	95.8 (PA169)	92.6
VP4	80	P[9]	95.6 (AU-1)	(AU-1)	P[9]	95.3 (AU-1)	(AU-1)	94.6
VP6	85	I2	86.9 (RF)	95.5 (Hun5)	I2	91.8 (RF)	92.4 (UKtc)	86
VP7	80	G3	90.1 (AU-1)	94.2 (Cat2)	G3	90.5 (AU-1)	95.9 (Cat2)	95.7
NSP1	79	A3	93.6 (AU-1)	94.8 (Chubut)	A3	92.9 (AU-1)	94.1 (Chubut)	92.1
NSP2	85	N1	91.4 (Wa)	99.1 (Cat2)	N2	90.1 (RF)	91.4 (NCDV)	79
NSP3	85	T6	94.7 (RF)	97 (MG6)	T6	95.9 (RF)	(RF)	97.5
NSP4	85	E2	87 (DS-1)	92.5 (PA169)	E2	87.5 (DS-1)	98 (PA169)	92.3
NSP5	91	H3	96.8 (RF)	97.3 (111/05)	H3	98 (RF)	98.8 (Cat2)	96.3

*Numeric values given as % nt. Percentage nucleotide cutoff values and genotype proposed by Matthijnssens et al. (*8*). VP, structural protein; NSP, nonstructural protein.
†Prototype genotype strains used by Matthijnssens et al. (*8*).
‡ Strains that shared the highest nucleotide identity in the cognate genes with the Italian G3P[9] rotaviruses.

sessed the following genetic constellations: G3-P[9]-I2-R2-C2-M2-A3-N1-T6-E2-H3 for strain PAH136/96 and G3-P[9]-I2-R2-C2-M2-A3-N2-T6-E2-H3 for strain PAI58/96, differing only in the NSP2 gene (Tables 1, 2). Phylogenetic analysis showed that the VP2, VP3, VP4, VP7, NSP1, NSP3, NSP4, and NSP5 genomic segments of the 2 Italian G3P[9] viruses were closely related to each other, sharing high sequence similarity (Table 1; online Technical Appendix, www.cdc.gov/EID/content/16/5/859-Techapp.pdf). Although included in the same VP1 (R2) and VP6 (I2) genotypes, the 2 G3P[9] viruses in the VP1 and VP6 trees showed distinct patterns of segregation (online Technical Appendix). Strains PAH136/96 and PAI58/96 shared only 83.2% nt and 86% nt identity in the VP1 and VP6 genes, respectively, i.e., values slightly above the proposed cutoff values for the VP1 (83%) and VP6 genotypes (85%) (Table 1).

We found that after sequence and phylogenetic analysis, each of the 11 genomic segments of the Italian G3P[9] viruses had a striking genetic similarity with the corresponding segment of G6/G8P[14] human or ruminant rotaviruses or to human/feline AU-1–like rotaviruses (Table 1; online Technical Appendix). In particular, the PAH136/96

Table 2. Complete genomic constellations of the 2 G3P[9] Italian viruses sequenced together with several human P[14], ruminant, and feline rotaviruses and reference human strains Wa, DS-1, and AU-1*

Strain	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
Hu/Ita/PA169	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Hu/Hung/Hun5	G6	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
Hu/B10925	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Hu/MG6	G6	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
Hu/111/05	G6	P[14]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Ov/OVR762	G8	P[14]	I2	R2	C2	M2	A11	N2	T6	E2	H3
Gu/Arg/Chubut	G8	P[14]	I2	R5	C2	M2	A3	N2	T6	E2	H3
Bo/RF	G6	P[5]	I2	R2	C2	M2	A3	N2	T6	E2	H3
Bo/NCDV	G6	P[1]	I2	R2	C2	M2	?	N2	T6	E2	H3
Hu/Ita/PAI58/96	**G3**	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	**N2**	**T6**	**E2**	**H3**
Hu/Ita/PAH136/96	**G3**	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	**N1**	**T6**	**E2**	**H3**
Fe/Cat-2	G3	P[9]	I3	R3	C2	M3	A3	N1	T6	E3	H3
Fe/Cat-97	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
Ca/CU-1	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
Ca/Ro1845	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
Ca/K9	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
Ca/A79–10	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
Hu/HCR3A	G3	P[3]	I3	R3	C2	M3	A9	N2	T3	E3	H6
Hu/AU-1	G3	P[9]	I3	R3	C3	M3	A3	N3	T3	E3	H3
Hu/DS-1	G2	P[4]	I2	R2	C2	M2	A2	N2	T2	E2	H2
Hu/Wa	G1	P[8]	I1	R1	C1	M1	A1	N1	T1	E1	H1

*Gray shading indicates genetic relationships with respect to the G3P[9] Italian viruses, according to the patterns of segregation displayed in the phylogenetic analyses in the online Technical Appendix (www.cdc.gov/EID/content/15/5/859-Techapp.pdf). **Boldface** indicates complete genomic constellations of the 2 G3P[9] Italian viruses sequenced in this study. VP, structural protein; NSP, nonstructural protein.

strain possesses VP1, VP2, VP3, VP6, NSP3, NSP4, and NSP5 gene segments closely related to G6P[14] human rotaviruses, and the PAI58/96 strain possesses VP1, VP2, VP3, VP6, NSP2, NSP3, and NSP4 gene segments clustering with P[14] human rotaviruses, bovine rotaviruses, and other ruminant rotaviruses. The NSP1 genes of both strains segregated in a distinct branch within the A3 genotype containing feline, human/feline-like rotaviruses, and a G8P[14] rotavirus strain isolated from a guanaco (online Technical Appendix). Matthijnssens et al. demonstrated that human G6P[14] rotaviruses were closely related to bovine and G6/G8P[14] ovine, antelope, and guanaco rotavirus strains because they shared a consensus genomic constellation (G6/G8)-P[14]-I2-(R2/R5)-C2-M2-(A3/A11)-N2-T6-(E2/E12)-H3 (*13*). The NSP2 gene of PAH136/96 and the NSP5 of PAI58/96 were strictly related to the cognate sequences of the feline Cat2 strain. The VP7 and VP4 genes of PAH136/96 and PAI58/96 human rotaviruses were also highly similar to the Cat2 strain and the human/feline-like AU-1 strain. The Cat2 strain displayed a puzzling genomic composition (G3/P[9]-I3-R3-C2-M3-A3-N1-T6-E3-H3), which we hypothesize resulted from multiple reassortment events involving canine, feline, human, and bovine rotaviruses (*14*). No genetic correlation was found in the VP7 gene between the G3P[9] strains of this study and the human G3 strains circulating in Palermo over a 20-year surveillance period (*15*).

Conclusions

Full-genome sequencing of 2 unusual G3P[9] human rotavirus strains identified in Italy indicated that 1) viruses with a genetic makeup different from the Wa-, DS-1-, and AU-1-like gene pools may circulate in humans; 2) these viruses appear to have a relatively stable genetic constellation originating from reassortment events among human/feline AU-1–like rotaviruses, feline Cat2-like rotaviruses, and either ruminant rotaviruses or G6P[14] human rotaviruses; 3) these viruses, although retaining a stable genetic constellation, do not appear to have a clonal origin but are more likely to result from multiple introductions of particular genome segments from currently unknown animal rotavirus reservoirs.

Investigating the genetic features of human rotaviruses with unusual genetic/antigenic makeup is pivotal to gather information on the mechanisms by which some rotavirus strains may emerge in human populations. In addition, because of the possible animal origin of G3P[9] viruses, epidemiologic studies are warranted to identify the animal reservoir.

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Transmission of Hemagglutinin D222G Mutant Strain of Pandemic (H1N1) 2009 Virus

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A pandemic (H1N1) 2009 virus strain carrying the D222G mutation was identified in a severely ill man and was transmitted to a household contact. Only mild illness developed in the contact, despite his obesity and diabetes. The isolated virus reacted fully with an antiserum against the pandemic vaccine strain.

On November 20, 2009, the Norwegian Institute of Public Health reported to the World Health Organization a mutation in the hemagglutinin (HA) of pandemic (H1N1) 2009 virus, consisting in a change of aspartic acid (D) with glycine (G) at aa 222. The mutation had been detected in 3 patients (the first 2 fatal cases in the country and in 1 patient with severe pneumonia) among ≈70 other patients with pandemic (H1N1) 2009, suggesting that it was not widespread in Norway (*1*). The same mutation has been also detected in Brazil, China, Japan, Mexico, Ukraine, the United States, France, and Spain (*1*,*2*).

The D-to-G mutation among the 1918 influenza virus variants (*3*) correlated with a shift from α2-6–linked sialic acid preference to a dual α2-3/α2-6 specificity. However, whether such a mutation may alter receptor binding specificity in the pandemic (H1N1) 2009 virus is unknown.

Although several pandemic (H1N1) 2009 viral strains sharing this mutation were detected in fatal cases, the same mutation also was detected in some mild cases; conversely, viruses from numerous fatal cases have not shown the same mutation. Thus, the clinical and public health significance of this finding remains unclear. The mutation appears to occur sporadically and spontaneously. No links between the small number of patients infected with the mutated virus have been found, and the mutation did not appear to spread (*4*). On the basis of results from a retrospective HA1 sequence analysis performed on pandemic (H1N1) 2009 viral isolates in Italy, we report on a transmission event of this virus carrying the D222G mutation.

The Study

We reexamined the HA sequences of 130 influenza A (H1N1) virus strains identified from patients affected by pandemic (H1N1) 2009. The neuraminidase sequences of some of these viruses also have been analyzed.

All 130 strains had been obtained from clinical samples (nasal, pharyngeal, or nasopharyngeal swabs and/or tracheal aspirates) collected during May–November 2009 in the context of virologic surveillance conducted by the National Influenza Centre, in collaboration with the regional laboratory network. These samples were obtained aimed to study the evolution of the pandemic strain. Forty-one HA gene sequences examined in the present study were retrieved from the National Center for Biotechnology Information, 3 from the Global Initiative on Sharing Avian Influenza Data database, and 86 HA sequences (47 directly from the clinical samples and 39 from cell culture supernatant) were obtained at the National Influenza Centre (NIC) with the following procedure. Viral RNAs were extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Santa Clara, CA, USA) and amplified by reverse transcription–PCR (RT–PCR) (*5*). HA amplicons were sequenced by using the BigDye Terminator Cycle-Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA) and ABI Prism 310 DNA sequencer (Applied Biosystems). All the NIC sequences were deposited in the GenBank database under the accession numbers reported in the Figure. Sequences were assembled and aligned using Lasergene package, version 4.0 (DNASTAR, Madison, WI, USA). BioEdit software version 4.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html) of the MEGA software package (www.megasoftware.net) was used to estimate phylogenies from the nucleotide sequences and to construct phylogenetic trees by using the neighbor-joining algorithm and maximum-likelihood method.

Among 130 patients, 23 of whom had severe disease (i.e., requiring hospitalization), only 1 was infected with a virus showing the D222G change. The patient, a man 25 years of age from northern Italy, had a febrile illness on August 17. One week later, he was admitted to an intensive care unit with severe pneumonia and acute respiratory distress syndrome, which resolved after treatment with

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[1]Members of this group are listed at the end of this article.

extracorporeal membrane oxygenation. Influenza A (H1N1) viral genome showing the D222G mutation was identified through direct sequencing of nasopharyngeal swab and tracheal aspirate collected on August 27. No sequence could be retrieved from a nasal wash, which was obtained the same day and was positive in real-time RT–PCR for pandemic (H1N1) 2009 virus. No viral growth was detected in MDCK cells seeded with both clinical samples.

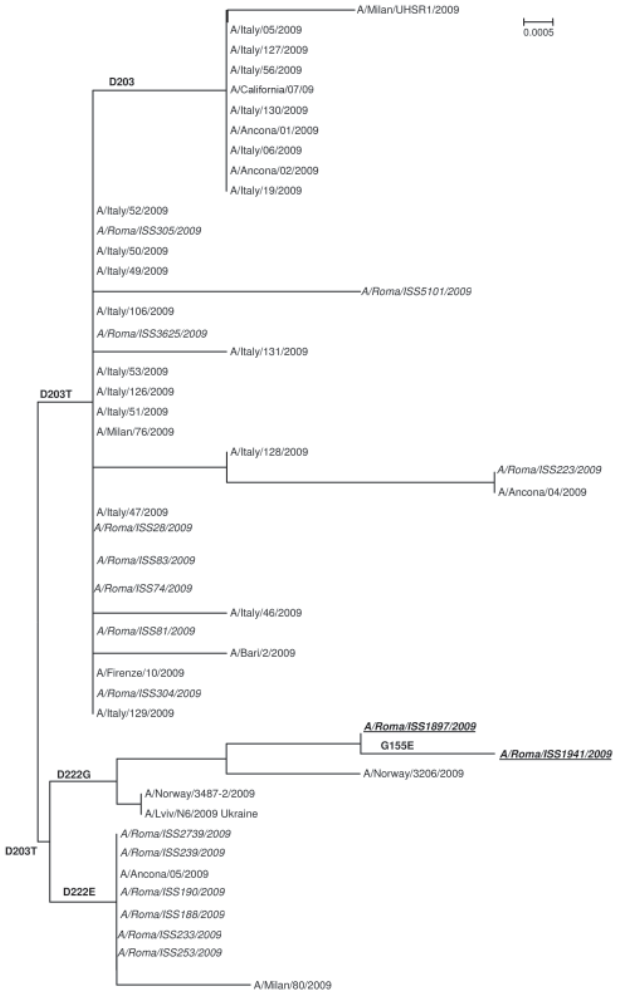

Figure. Phylogenetic relationships of hemagglutinin (HA) 1 sequences of pandemic (H1N1) 2009 viruses in Italy obtained from the National Influenza Centre (NIC)–Istituto Superiore di Sanità (ISS) and the National Center for Biotechnology Information. The NIC-ISS sequences are in *italics*. Isolates from the index case-patient and his father are indicated in **boldface**. Amino acid mutations of interest in this study are reported on the nodes. All the NIC sequences obtained in the present study were deposited in the GenBank database under the following accession numbers: (hemagglutinin sequences) GU451262–GU451280; GU576500–GU576502; GU576504; GU576506; GU576508; GU576510; GU576512; GU576514-GU57615; GU576517; GU576519; GU576521-GU576522; GU576524; GU576526-GU576527; GU576529; GU576531-GU576532; GU576534; GU576536-GU576540; GU585403–GU585443; (neuraminidase sequences) GU936490-GU936491. Scale bar indicates proportion of nucleotide substitutions per site.

To identify possible transmission chains of the mutated virus, we analyzed the genome of the viral strain detected in the throat swab of the father of the index case-patient, 55 years of age, who was obese and had diabetes. He became moderately ill on August 25 but did not require hospitalization or antiviral treatment. The virus isolated in MDCK cells from the sample obtained on August 27 had the same HA mutation, D222G. Viral strains from index and contact cases were susceptible to oseltamivir, as determined by lack of the specific oseltamivir-resistance marker (His274Tyr, N2 numbering) in neuraminidase sequences, which were identical in both viral strains. Additional samples analyzed from close contacts (i.e., 4 healthcare workers, 4 family members, and 2 friends) of the 2 patients all were negative for pandemic (H1N1) 2009 virus.

Comparison of the 2 HA1 sequences from the index case-patient and his father showed an additional substitution in the latter (G155E), which is located close to the receptor-binding pocket. Furthermore, sequence analyses showed that all virus strains containing a change in HA1 position 222 (D222G or D222E) also showed a second substitution in position 203 (D203T), when compared with the A/California/7/2009 vaccine strain (Figure). However, the effect of this second mutation on the HA receptor-binding properties is still unclear. The HA1 genes from the index and contact case-patients shared 2 additional nucleotide changes, 1 synonymous substitution (T504C, N1 numbering) not found in any other sequence analyzed in the present study and another nucleotide change resulting in an amino acid substitution (P297S, N1 numbering), detected only in a small number of sequences from Italy. Hemagglutination-inhibition test of the isolated virus did not show substantial reduction in its reactivity with an antiserum against pandemic (H1N1) 2009 vaccine, as compared with the reactivity of an influenza virus A/California/7/2009 strain not carrying the G155E mutation (Table).

Conclusions

Identification of a pandemic (H1N1) 2009 virus strain carrying the D222G mutation and its association with the first fatal cases of influenza in Norway raised some concern about emergence of a viral strain with increased pathogenicity. No data have been reported on transmission capacity of this and other D222G variants, occasionally identified worldwide. Our findings suggest that the D222G mutated virus is to some extent transmissible. However, a number of close contacts were identified who did not acquire the infection.

Whether the mutated virus may have a lower fitness for receptors in the high respiratory tract, which may affect transmission, remains undefined. Furthermore, the muta-

Table. Hemagglutination-inhibition test results of pandemic (H1N1) 2009 viruses, Italy

Viruses	Postinfection ferret serum titer against	
	A/Brisbane/59/2007 seasonal H1N1	A/California/7/2009 pandemic H1N1
A/Brisbane/59/2007 (H1N1)	1,280	<10
A/California/7/2009 (H1N1v)	<10	1,280
Father's virus isolate (D222G + G155E)	<10	640
A/Rome/ISS11/2009	<10	320

tion, which was first found in a severely affected man, was transmitted to a family member (father), who had only a mild illness, despite risk for severe infection. The HA1 sequence from this latter case presented an additional amino acid substitution (G155E), which was not found in any other sequence analyzed in our study and rarely detected among all sequences available from GenBank. Recent data suggest that amino acid substitution at this position may be critical for the switch from dual α2-3/α2-6 binding specificity to α2-6 linkage (*6*), which is preferentially recognized by human influenza viruses and expressed mainly in the upper respiratory tract. This change might be partially responsible for the milder influenza illness developed by the father of the index case-patient. In addition, the G155E mutation in laboratory-generated variants of pandemic (H1N1) 2009 virus has been associated with loss of antigenicity (*7*). In our study, the natural isolates of the virus carrying both the G155E and the D222G mutations had comparable antigenicity with the A/California/7/09-vaccine strain. Finally, our data do not support the association of the D222G mutation with severe disease.

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Schistosomiasis among Recreational Users of Upper Nile River, Uganda, 2007

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After recreational exposure to river water in Uganda, 12 (17%) of 69 persons had evidence of schistosome infection. Eighteen percent self-medicated with praziquantel prophylaxis immediately after exposure, which was not appropriate. Travelers to schistosomiasis-endemic areas should consult a travel medicine physician.

Schistosomiasis, a parasitic infection caused by schistosome flukes, affects 207 million persons worldwide, mostly in sub-Saharan Africa (*1*). Schistosomiasis has been reported among travelers (*2*–*12*); 3 outbreaks have been reported among white-water rafters on the Omo River in Ethiopia (*2*,*7*,*10*). During September–November 2007, the Centers for Disease Control and Prevention (CDC) received reports of schistosome infection among travelers returning from white-water rafting on the Nile River, Jinja District, Uganda. Approximately 12,000 persons raft each year in Uganda, and local rafting companies believe that exposure to fast-moving white water during rafting and kayaking presents a low risk for schistosomiasis (C. McLeay, pers. comm.).

The Study

During November 30–December 5, 2007, we enrolled a convenience sample of competitors and spectators attending the international Nile Freestyle Festival kayaking event and tourists on commercial rafting trips in Jinja District, Uganda. We administered a questionnaire to collect information about participants' demographic characteristics, use of praziquantel (the antiparasitic drug treatment for schistosome infection), and exposure to fresh water. Three months after enrollment, we asked study participants who had had a negative or indeterminate result from a blood test for schistosome antibodies at the time of enrollment to complete an Internet-based questionnaire about freshwater exposures, health symptoms, and medical tests or treatments for schistosomiasis since enrollment.

We measured infection by collecting two 5-mL blood samples 3 months apart and testing them for evidence of schistosome antibody seroconversion. We tested for presence of schistosome-specific antibodies using an ELISA assay screening test that is 99% sensitive for *Schistosoma mansoni* and 90% sensitive for *S. hematobium* (*10*). We confirmed FAST-ELISA–positive samples using an *S. mansoni*–specific immunoblot to detect species-specific antibody. We tested all samples using an *S. hematobium*–specific immunoblot, which is 95% sensitive and 99% specific for each species (*13*). We defined a positive test result as positive results by both tests, an indeterminate result as positive by FAST-ELISA but negative by immunoblot, and a negative result as negative by both tests.

We defined study participant exposures from 2 weeks before enrollment until second sample collection by 4 activity categories: no water-contact activity, swimming/wading only, kayaking/rafting only, and swimming/wading plus kayaking/rafting. We defined schistosome antibody seroconversion in participants as either being first-test–negative and second-test–positive, or being first-test–negative and second-test–indeterminate. We compared characteristics between groups using the χ^2 test for categorical data and the Mann-Whitney test for continuous variables (*14*). We expressed the risk for infection as the proportion of persons in each activity category who had evidence of schistosome antibody seroconversion and calculated the Mantel-Haenszel χ^2 test for trend (*14*). We performed all analyses using SAS version 9.1 (SAS Institute, Cary, NC, USA). The CDC Institutional Review Board and the Uganda Virus Research Institute approved this study.

We enrolled 150 study participants; 2 subsequently withdrew. Thirty-five (24%) participants were not followed up because their first blood test was positive; all of these persons reported previous exposure to fresh water in schistosomiasis-endemic countries. Of the remaining 113 persons eligible for follow-up, 69 (61%) provided a second blood sample. Persons who provided only 1 blood sample were more likely to be younger ($p = 0.005$) and female ($p = 0.03$) (Table 1).

Of 69 persons followed up, 23% had fever, 13% cough, 10% skin rash, and 10% abdominal pain; 8% reported prickling skin. None reported physician-diagnosed acute schistosomiasis. Twelve (17%) of the 69 persons with 2 blood samples had evidence of seroconversion. No

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Table 1. Characteristics of 113 recreational users of the upper Nile River who participated in a study to determine risk for schistosome infection, were eligible for study follow-up, and provided 1 or 2 blood samples, Uganda, 2007*

Characteristic	Provided 1 blood sample, no. (%), n = 44	Provided 2 blood samples, no. (%), n = 69	p value†
Male sex	14 (32)	34 (50)	0.03
Main reason for being at the Nile			
Rafter	20 (45)	32 (46)	0.4
Spectator at the competition	12 (27)	18 (26)	
Kayak competitor	9 (21)	8 (12)	
Other	3 (7)	11 (16)	
Region of residence			
Africa	6 (14)	16 (23)	0.6
Americas	12 (27)	16 (23)	
Europe	15 (34)	17 (25)	
Australasia	7 (16)	12 (17)	
None given	4 (9)	8 (12)	

*Median ages (ranges) of study participants were as follows: 1 blood sample provided: 25 y (18–41 y); 2 blood samples provided, 29 y (16–67 y); p = 0.005.
†p values estimated by using the Mann-Whitney test for age and a χ^2 test for all other variables.

seroconversions were identified among the 9 persons who reported no water-contact activities. Serologic data suggested that infection occurred in 1 (13%) of 8 reporting swimming/wading only; 4 (15%) of 26, kayaking/rafting only; and 7 (27%) of 26, swimming/wading plus kayaking/rafting (Table 2).

Of 106 persons for whom data were recorded, 19 (18%) reported self-medicating with praziquantel while at the kayaking competition. Of the 12 participants with evidence of seroconversion, 6 had data recorded about self-medication, none of whom took praziquantel.

Conclusions

Approximately one fifth of persons with recreational exposure to water on the upper Nile River in Jinja District showed evidence of schistosome antibody seroconversion. Infection occurred among persons who reported swimming/wading only, kayaking/rafting only, and both activities, which refutes the belief that exposure to fast-moving water presents a low risk for schistosomiasis.

Exposure to schistosomes is likely to be highest in slow-moving water near riverbanks; thus, persons who go rafting may be at highest risk while putting their kayaks/rafts into and taking them out of the river. Although we were unable to estimate the risk for infection attributable to fast-moving white-water exposure alone, we did find that persons who reported swimming/wading and kayaking/rafting had the highest risk, possibly because of increased duration of exposure (*4*).

Table 2. Proportion of recreational users of the upper Nile River who had schistosome infection, Uganda, 2007*

Activity	No. infected/total (%)
No water-contact activity	0/9
Swimming/wading only	1/8 (13)
Kayaking/rafting only	4/26 (15)
Kayaking/rafting and swimming/wading	7/26 (27)
All study participants	12/69 (17)

*Activity categories are mutually exclusive. χ^2 test for trend p = 0.06

Eighteen percent of study participants reported self-medicating with praziquantel immediately or shortly after river water exposure. However, they would not have been protected against schistosomiasis because praziquantel acts against mature schistosome parasites and thus is most effective if taken after the parasite has developed to the adult stage, which is 4–6 weeks after infection. Local advice about using praziquantel to prevent schistosomiasis may not be appropriate; because indigenous populations have ongoing exposure, timing of treatment is not as critical. Travelers with discrete freshwater exposures in schistosomiasis-endemic countries should consult a travel medicine physician. In addition, information could be made available to pharmacies, rafting companies, and travelers about when to take praziquantel.

Our study had several limitations. The study cohort was a convenience sample, and participants might not have had equal chance of being enrolled. Use of this sample may have introduced bias, although whether any such bias would contribute to overestimation or underestimation of risk is unclear. Because schistosome antibody tests do not differentiate newly acquired infection, we excluded persons with first-test–positive results from the study follow-up. However, if these persons were more likely to have had a higher risk for infection, excluding them would have led us to underestimation risk for infection.

More than 12,000 persons take rafting trips in Uganda each year. Many travelers do not follow advice to avoid freshwater activities in schistosomiasis-endemic countries (*15*). Travelers should be made aware that white-water exposure presents a risk for schistosomiasis and that treatment with praziquantel should be at least 4–6 weeks after last exposure, preferably under the direction of a travel medicine physician.

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Kobuvirus in Domestic Sheep, Hungary

To the Editor: Picornaviruses (family *Picornaviridae*) are small, nonenveloped viruses with single-stranded, positive-sense genomic RNA. They are divided into 12 genera: *Enterovirus, Aphthovirus, Cardiovirus, Hepatovirus, Parechovirus, Erbovirus, Teschovirus, Sapelovirus, Senecavirus, Tremovirus, Avihepatovirus*, and *Kobuvirus*. The genus *Kobuvirus* consists of 2 officially recognized species, *Aichi virus* (*1*) and *Bovine kobuvirus* (*2*), and 1 candidate species, porcine kobuvirus (*3*). The kobuvirus genome is ≈8.2–8.4 kb long and has the typical picornavirus genome organization of leader (L) protein following the structural (viral protein [VP] 0, VP3, and VP1) and nonstructural (2A–2C and 3A–3D) regions (*2*,*4*). The genetic identity on the coding region between Aichi (strain A846/88), bovine (U-1), and porcine (S-1-HUN) viruses is between 35% (L protein) and 74% (3D region) (*2*,*4*).

Aichi virus and bovine kobuvirus were first detected in fecal samples from humans and cattle in Japan, in 1991 and 2003, respectively (*1*,*2*). Porcine kobuvirus was identified from domestic pigs in Hungary in 2008 (*3*,*4*). Recent studies demonstrated that Aichi virus circulates in Asia (*5*), Europe (*6*,*7*) including Hungary (*4*), South America (*6*), and North Africa (*8*) and can cause gastroenteritis in humans. In addition, bovine and porcine kobuviruses are detected among these farm animals in Europe (*4*) and Asia (*2*,*9*). These data indicate that kobuviruses are widely distributed geographically and raise the possibility of additional animal host species. We detected kobuvirus in sheep.

On March 17, 2009, a total of 8 fecal samples were collected from young, healthy, domestic sheep (*Ovis aries*) <3 weeks of age in a herd of 400 animals in central Hungary. At this farm, merino ewes from Hungary were mated with blackhead meat rams from Germany. At the time of sampling, no clinical signs of diarrhea were reported. Reverse transcription–PCR was performed by using generic kobuvirus screening primers (UNIV-kobu-R/F) reported previously (*4*). These primers were designed for Aichi virus (GenBank accession no. AB040749), bovine (AB084788), and porcine kobuvirus (EU787450) sequences and amplify a 216-nt region of 3D (RNA-dependent RNA polymerase region). The continuous 3D and 3′ untranslated regions (UTRs) of the kobuvirus genome in sheep were determined by using the 5′/3′ RACE (rapid amplification of cDNA ends) kit, 2nd generation (Roche Diagnostics GmbH, Mannheim, Germany) and primers UNIV-kobu-F and S-1-F-7518/7540 (5′-CACTTCCATCATCAACACCATCA-3′ corresponding to nt 7518–7540 of bovine kobuvirus) (*4*). PCR products were sequenced directly in both directions by using the BigDye Reaction Kit (Applied Biosystems, Warrington, UK) with the PCR primers and sequenced by an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Stafford, TX, USA). Phylogenetic analysis was conducted by using MEGA version 4.1 (www.megasoftware.net). The sequence for kobuvirus/sheep/TB3-HUN/2009/Hungary was submitted to GenBank under accession no. GU245693.

Of the 8 sheep fecal samples, 5 (62.5%) were positive for kobuvirus. The partial 3D region (216 nt) was genetically identical for all 5 strains. The 3′ continuous nucleotide sequence of the partial 3D (688 nt) and 3′ UTR (174 nt) regions of strain kobuvirus/sheep/TB3-HUN/2009/Hungary(TB3-HUN; GU245693) was determined. TB3-HUN had 59%–66% (862) nt and 77%–84% aa identities to Aichi and porcine kobuviruses, respectively. Strain TB3-HUN had 89/97% nt/aa and 86% nt identities to bovine kobuvirus in the 3D/3′ UTR (862 nt) and 3′ UTR (174 nt) regions, respectively. Phylogenetic analysis of the overlapping partial 3D/3′ UTR nucleotide sequence of TB3-HUN from sheep and of reference bovine, porcine, and human kobuviruses confirmed that ovine kobuvirus strain TB3-HUN is related to bovine kobuviruses (Figure).

The nucleotide sequence of the partial 3D/3′ UTR region of kobuvirus in sheep has high nucleotide identity to bovine kobuviruses and forms the same lineage (but a different sublineage) with the kobuvirus strains in cattle. This result raised the following questions: can a highly similar kobuvirus be present in (and pathogenic for) 2 animal species (cattle and sheep), or is this result a consequence of natural contamination? The concept of sheep as host is supported by the high prevalence of kobuvirus in young healthy sheep; by the sublineage position of the sheep strain on the phylogenetic tree according to the most conserved genetic region; and by the genetic relation between the 2 potential ruminant hosts, cattle and sheep. The existence of 1 pathogen in 2 host species (cattle and sheep) is well known, e.g., for bluetongue virus, adenoviruses, ovine herpesvirus type 2, and foot-and-mouth disease picornaviruses (*10*). Alternatively, the possibility of natural contamination cannot be excluded. The possibility of passive virus shedding in sheep exists because a cattle farm was located next to the tested sheep herd and would enable fecal–oral transmission of kobuvirus between these farm animals. Both possibilities (host and passive virus reservoir) are preliminary perceptions, regardless which is true. Further molecular and epidemiologic studies are required to determine the relevance, distribution, and diversity of kobuvirus or kobuviruses in sheep.

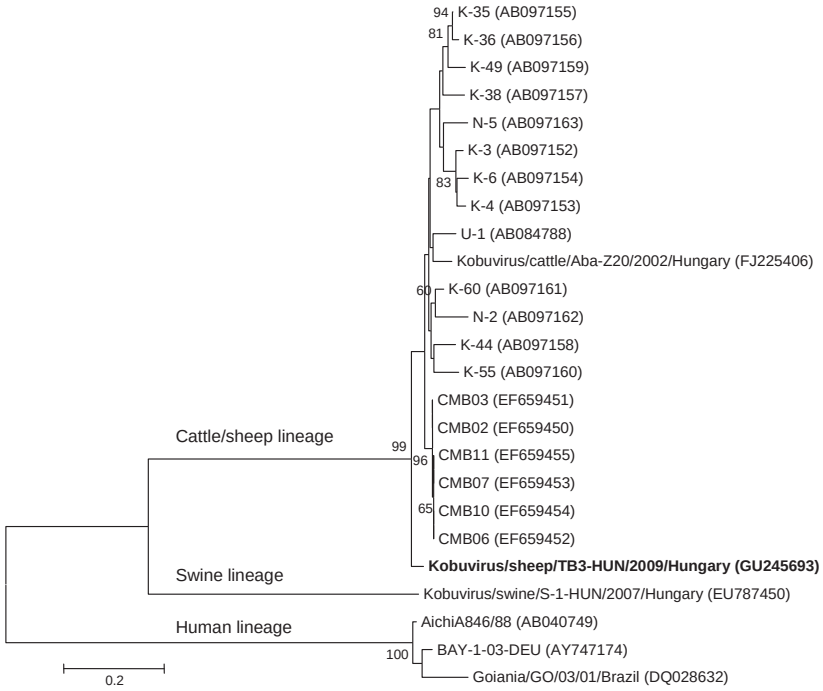

Figure. Phylogenetic analysis of kobuvirus in sheep (kobuvirus/sheep/TB3-HUN/2009/Hungary, GU245693) and kobuvirus lineages in humans, cattle, and swine, according to the 862-nt fragment of the kobuvirus 3D/3′ untranslated regions. The phylogenetic tree was constructed by using the neighbor-joining clustering method with distance calculation and the maximum-composite likelihood correction for evolutionary rate with MEGA version 4.1 software (www.megasoftware.net). Bootstrap values (based on 1,000 replicates) are given for each node if >50%. Reference strains were obtained from GenBank. **Boldface** indicates virus detected in sheep. Scale bar indicates nucleotide substitutions per site.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Physician Awareness of Chagas Disease, USA

To the Editor: The year 2009 was the 100th anniversary of Carlos Chagas's discovery of the disease caused by the parasite *Trypanosoma cruzi*, now known as Chagas disease. Spread by infected bugs called triatomines, the disease is endemic throughout much of Mexico and Central and South America. An estimated 8–11 million persons in Latin America have the disease (*1*). *T. cruzi* infection causes more public health problems with long-term consequences in Latin America than any other parasitic disease (*2*).

Although earlier estimates suggested that 100,000 infected persons may live in the United States (*3*), recent data suggest that ≈300,000 persons are infected (*4*). Most of these persons are unaware that they are infected. Antiparasitic drugs to treat Chagas disease have not been approved by the US Food and Drug Administration and are only available in the United States through the Centers for Disease Control and Prevention (CDC) for use under investigational protocols for compassionate treatment.

Screening of the US blood supply for Chagas disease began in early 2007; more than 500 donors with *T. cruzi* infection were identified within the first 18 months (*5*). However, to date, only ≈11% of *T. cruzi*–positive donors (or their physicians) have contacted CDC for treatment consultations (CDC, unpub. data). One possible explanation is the limited awareness of Chagas disease in the United States among health professionals, the general public, and even among Mexican-born Americans (K.K. Stimpert and S.P. Montgomery, unpub. data).

To assess healthcare provider awareness of Chagas disease, a study was conducted by MedscapeCME (www.medscape.com) with technical support from CDC. MedscapeCME, a leading provider of online clinical and medical information and continuing medical education (CME) for physicians and other healthcare professionals, carried out a knowledge, attitudes, and practices (KAP) assessment of physician members of its website. Researchers anticipated that the results of the study would inform larger formal studies among healthcare providers in geographic areas where population demographics and blood donor screening results suggest that persons are at risk for Chagas disease.

The KAP study was based on five 10-question surveys, which were posted on the MedscapeCME website. Each survey was tailored for a specific specialty: primary care, infectious disease, cardiology, obstetrics/gynecology, and transplantation medicine; they were designed to measure basic knowledge of Chagas disease epidemiology and consideration of Chagas disease risk among these physicians.

MedscapeCME members were given the option of participating in the surveys when they logged onto each specialty site. MedscapeCME membership requirements include online registration, which is validated by confirming American Medical Association membership. Each member could complete the survey only once. All surveys were launched on December 11, 2008, but discontinued at different times (median 39 days).

Although familiarity with Chagas disease varied by specialty, the results suggested that a general lack of awareness was common across all groups (Table). This awareness deficit was most pronounced in obstetricians and gynecologists and least pronounced in infectious disease physicians (Table). The 7 responses to questions outlined in the Table may be placed in 3 categories: 1) general awareness of Chagas disease (questions 1 and 3), 2) confidence in Chagas disease knowledge and consideration of risk (questions 2 and 5), and 3) clinical aspects of Chagas disease (questions 4, 6, and 7). Across all 3 domains, obstetricians and gynecologists reported low knowledge of Chagas disease and a low level of confidence in their knowledge. Infectious disease physicians had the highest levels of knowledge and confidence across all domains, except for responses to question 1 (Table).

Survey results suggest substantial knowledge deficits among physicians, especially among obstetricians and gynecologists. The apparent lack of knowledge in the obstetrics and gynecology community is of particular concern because Chagas disease can be transmitted congenitally (*6*). Because Chagas disease may also be transmitted by organ transplantation, the proportion of respondents from this specialty who indicated they never consider risk for Chagas disease in their patients (39%) is also notable. In fact, many physicians surveyed never consider the risk for Chagas disease in their patient population (29%–60%) and are not at all confident that their knowledge of Chagas disease is current (27%–68%).

The study has some limitations. The conclusions are drawn from a study in which convenience samples from selected populations were used and are thus not generalizable to larger healthcare provider populations in the United States. The high participation rate of transplant surgeons may reflect a fluctuation in membership numbers. In addition, transplant surgeons may have an increased interest in Chagas disease because of recent instances of transplant-associated transmission (*7*,*8*).

These preliminary data suggest a substantial knowledge deficit regarding Chagas disease among healthcare providers, which could have a negative effect on patient health if Chagas disease is not recognized and appropriately treated. CDC plans to conduct larger scale KAP surveys of physicians in areas where blood donor screening results suggest relatively high preva-

Table. Reponses to MedscapeCME knowledge, attitudes, and practices survey questions reflecting lack of knowledge about Chagas disease, by specialty*

Response	No. (%) respondents†				
	Cardiology, n = 280	Infectious disease, n = 167	OB/GYN, n = 292	Primary care, n = 278	Transplantation, n = 125
Never heard of Chagas disease‡	63 (23)	31 (19)	138 (47)	38 (14)	35 (25)
Not at all confident of Chagas disease knowledge being up to date§	87 (44)	31 (27)	86 (68)	101 (47)	41 (48)
Did not know parasite causes Chagas disease¶	21 (16)	6 (5)	42 (33)	35 (16)	15 (17)
Did not know cardiac and/or gastrointestinal disease are manifestations of Chagas disease#	15 (8)	10 (9)	38 (30)	24 (11)	11 (13)
Never considers risk for Chagas disease in patients**	51 (34)	30 (29)	66 (60)	83 (43)	29 (39)
Did not know in what percentage of patients with chronic infection clinical disease develops††	66 (37)	30 (28)	60 (56)	93 (48)	35 (47)
Did not know Chagas disease symptoms‡‡	41 (23)	15 (14)	53 (48)	52 (27)	22 (29)

*OB/GYN, obstetrics/gynecology.
†Percentages are calculated based on number of respondents per question, by specialty.
‡Question 1: Have you heard of Chagas disease? Answer choices: yes, no.
§Question 2: How confident are you that your Chagas disease knowledge is up to date? Answer choices: very confident, confident, somewhat confident, not at all confident.
¶Question 3: Chagas disease is caused by a ___? Answer choices: bacterium, virus, parasite, fungus, I don't know.
#Question 4: People with chronic Chagas disease may have (check all that apply)? Answer choices: cardiac conduction abnormalities, cardiomyopathy, megacolon, co-clinical manifestations, I don't know.
**Question 5: How often do you consider the risk for Chagas disease in your patient population? Answer choices: never, rarely, sometimes, frequently, always.
††Question 6: Approximately what percentage of patients with chronic Chagas infection eventually develop clinical disease? Answer choices: <20%, 21%–40%, >40%, I don't know.
‡‡Question 7: Chagas disease symptoms are? Answer choices: Acute for several weeks then immediately symptomatic, Acute for several weeks asymptomatic for years to decades then sometimes symptomatic, There are no symptoms of Chagas disease, I don't know.

lence in the underlying communities. For example, *T. cruzi* infection prevalence in blood donors in Florida and California was reported to be 1/3,700 and 1/8,300, respectively (*5*). Future studies will help document why Chagas disease is underrecognized in the United States and further demonstrate the need for educating healthcare providers about this disease.

Acknowledgments

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Possible Transmission of Pandemic (HIN1) 2009 Virus with Oseltamivir Resistance

To the Editor: In March 2009, a new strain of influenza A (H1N1) virus of swine origin emerged; the virus had crossed the species barrier to humans and acquired the capability of human-to-human transmission. Soon after, the World Health Organization raised the worldwide pandemic alert to level 6 (www.who.int/en), declaring the first influenza pandemic in the past 42 years. The virus was named influenza A pandemic (H1N1) 2009 virus. The illness caused by this virus is particularly dangerous for pregnant women and for patients with chronic diseases (*1*). The preferred treatment is a neuraminidase inhibitor, zanamivir or oseltamivir (*2*).

Around the world, several dozen cases of resistance to oseltamivir in persons with or without exposure to the drug have been reported (*3*). However, only limited information is available with regard to initial infections with oseltamivir-resistant viruses (*4*). We report a case of possible human-to-human transmission of pandemic (H1N1) 2009 virus in Israel.

After the recent discovery of oseltamivir-resistant strains, we conducted a retrospective study of oseltamivir-resistance mutations in viral RNA amplified from specimens from patients hospitalized >1 week with pandemic (H1N1) 2009. All samples were first tested for the H275Y mutation by using an in-house real-time reverse transcription–PCR (RT-PCR) assay developed at the Central Virology Laboratory of Chaim Sheba Medical Center; positive results were confirmed by sequencing. The histidine-to-tyrosine mutation at the 275 position of the neuraminidase protein results in reduced binding of oseltamivir.

During June–August 2009, ≈80 children in an institution for disabled children were suspected of being infected with pandemic (H1N1) 2009 virus. The children had influenza-like signs and symptoms, and at that time the only influenza virus circulating in Israel was pandemic (H1N1) virus. Of these 80 patients, 10 were hospitalized because of the severity of their clinical signs or disease complications, and for 7, RNA of the pandemic (H1N1) 2009 virus was detected in throat and nasal swabs by real-time RT-PCR.

Patient 1 was a 13-year-old boy with cerebral palsy and partial blindness, who was treated with oseltamivir (60 mg twice a day) for 5 days (July 27–31, 2009). After some improvement, his condition worsened, and he was hospitalized on August 13 for breathing difficulty and high fever. Real-time RT-PCR indicated infection with pandemic (H1N1) 2009 virus. During our survey, we found that patient 1 was infected with a virus carrying the H275Y mutation, suggesting that the mutation evolved during oseltamivir treatment.

Patient 2 was a 10-year-old girl who had lived in the room next to patient 1 and who also had cerebral palsy. Her signs and symptoms started on August 13, 2009, and she was hospitalized on August 15. She was treated in the hospital for 5 consecutive days with oseltamivir, steroids, and augmentin; she was discharged on August 21. Her diagnosis was made early in the clinical course of her infection, and she was infected with pandemic (H1N1) 2009 virus carrying the H275Y mutation.

In contrast, none of the other 8 children who were hospitalized for pandemic (H1N1) 2009 carried the mutation. Although we cannot rule out the possibility that the virus was transmitted by a third person, we suggest that the virus carrying the resistance mutation was probably transmitted from patient 1 to patient 2. This transmission is probable because these 2 patients lived in adjacent rooms, they were in contact with each other, clinical onset of patient 1 preceded that of patient 2 by a few days, and patient 2 had the mutation at the beginning of her disease.

Fortunately, despite the conditions that favor virus spread in such institutions, this virus did not seem to spread further; the other 8 hospitalized children from this institution were infected with the wild-type virus. Nevertheless, the potential for spread of pandemic (H1N1) 2009 virus carrying the oseltamivir resistance mutation exists, thereby emphasizing the urgent need for a vaccination to prevent illness and for alternative drugs to treat infected patients.

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Cross-Reactive Antibodies to Pandemic (H1N1) 2009 Virus, Singapore

To the Editor: Accumulating evidence suggests that the degree of serologic cross-reactivity to pandemic (H1N1) 2009 virus varies between populations worldwide. To assess potential serologic cross-reactivity in Singapore, we obtained serum samples during May–June 2009 from 50 randomly recruited, healthy volunteers born mostly before 1958 (i.e., potentially those with some natural exposure to the then circulating H1N1/1918-like subtype viruses) before widespread transmission of pandemic (H1N1) 2009 virus in Singapore. Standard serologic hemagglutination-inhibition (HI) tests (*1*) were performed in 2 reference laboratories (Singapore during July–October 2009 and Melbourne, Australia, in January 2010), and microneutralization (MN) tests (*2*) were performed in 1 reference laboratory (Singapore) for each serum sample against pandemic (H1N1) 2009 virus (A/Auckland/1/2009) and seasonal influenza (H1N1) virus (A/Brisbane/59/2007). The study was reviewed and approved by the National Healthcare Group Domain-Specific Review Board (ref no. E/09/289, J.W.T. principal investigator).

Mean ± SD age of participants was 60.1 ± 7.4 years (range 45–82 years); 31 (62%) were women, 42 (84%) were born in Singapore (the rest in Hong Kong, Malaysia, or India), and 26 (52%) had not traveled outside Singapore. None of the 50 participants had HI or MN titers ≥40 against influenza A/Auckland/1/2009 when samples were tested in either laboratory. In contrast, 18 samples had either HI or MN titers ≥40 against seasonal influenza A/Brisbane/59/2007 (Table). Use of guinea pig or turkey erythrocytes in HI assays had little effect on the results (Table). Thus, our results are similar to those of Chen et al. (*3*) and Itoh et al. (*4*) for this small cohort in that none of the participants 40–80 years of age from Southeast Asia had cross-reactive antibodies to pandemic (H1N1) 2009 virus.

Although differences in population demographics and laboratory methods used make comparisons between studies difficult, one of the most striking observations from various studies has been the higher levels of cross-reactive antibody titers in prepandemic serum samples from older persons (≥80 years of age) in western populations (United States and United Kingdom) (*5*,*6*) than from persons in eastern populations (China) (*3*) and Singapore (this study). Although Itoh et al (*4*) did not find serologic cross-reactivity in the population <80 years of age in Japan, they found higher levels of cross-reactive antibodies in their population ≥80 years of age. Historically, because epidemiologic data suggest that influenza (H1N1)/1918–like viruses were widespread in Asia, these contrasting results are a stimulus for additional large-scale studies to assess the effect of these viruses in these populations.

Although the main limitation of our study is the small sample size, several reasons may account for different findings in population studies of serologic cross-reactivity. First, populations may not be comparable in terms of geographic proximity and their potential for community-acquired infection within the same wave of a seasonal influenza epidemic with a virus that was similar to pandemic (H1N1) 2009 virus. Chen et al. (*3*) reported that their serum samples were obtained mainly from rural farmers in China who lived farther apart than city dwellers, However, Hancock et al. (*5*) reported that their samples were obtained from vaccine trials conducted in 1976 or 2005–2009 involving academic, government, and industrial workers, which likely indicates that these persons were urban-based (i.e., living and working more closely to each other than rural farmers in China). Thus, results of our study may not be directly comparable with either of these previous studies because our population resided in Southeast Asia and was urban-based.

Second, use of seasonal influenza vaccine has varied in different populations, with Singapore having one of the lowest recorded use rates in the Western Pacific region, and far lower than that in the United States (*6*). If previous seasonal influenza viruses shared a degree of antigenic cross-reactivity with pandemic (H1N1) 2009 virus, contemporary seasonal influenza vaccines, if well-matched, should reflect changing antigenicity of seasonal influenza viruses; thus, vaccinated populations may have acquired some serologic cross-reactivity through previous influenza vaccines. However, it is likely that past infection rather than vaccination results in cross-reactivity, as suggested by Miller et al. (*7*).

Third, because pandemic (H1N1) 2009 virus originated mainly from swine viruses in North America and

Table. Cross-reactive antibody titers to pandemic (H1N1) 2009 and seasonal influenza viruses for 50 persons, Singapore, May–October 2009*

Patient age, y/sex	Laboratory 1 (Singapore General Hospital)				Laboratory 2 (WHO, Melbourne)+		
	A/Auckland/1/2009		A/Brisbane/59/2007		A/Auckland/1/2009		A/Brisbane/59/2007
	HI†	MN	HI†	MN	HI‡	HI†	HI‡
45/F	<10	<10	<10	<10	<10	<10	<10
52/F	<10	10	10	10	<10	10	<10
52/F	<10	<10	10	<10	<10	<10	<10
52/F	<10	<10	10	<10	<10	<10	<10
53/M	<10	<10	10	<10	<10	<10	<10
53/F	<10	<10	20	40	<10	<10	40
53/F	<10	<10	160	80	<10	<10	160
53/M	<10	<10	10	<10	<10	<10	<10
53/F	<10	<10	160	320	<10	<10	160
53/F	<10	<10	10	<10	<10	<10	<10
54/F	<10	<10	10	<10	<10	<10	<10
54/M	10	<10	10	<10	<10	20	<10
55/F	<10	<10	10	<10	<10	10	<10
55/F	<10	<10	160	40	<10	<10	160
55/M	<10	<10	10	<10	<10	<10	<10
56/F	<10	<10	20	10	<10	<10	10
56/F	<10	<10	80	80	<10	20	40
56/F	<10	10	10	40	<10	<10	10
56/F	<10	<10	10	<10	<10	<10	<10
57/M	10	20	40	40	20	20	<10
58/M	<10	<10	20	20	<10	10	20
58/F	<10	<10	40	20	<10	<10	80
59/M	<10	<10	10	<10	<10	<10	<10
59/M	<10	<10	20	20	20	20	10
59/F	<10	<10	40	40	<10	<10	40
60/M	<10	<10	80	40	<10	<10	80
60/M	<10	<10	20	10	<10	<10	20
60/F	10	<10	10	<10	NR	<10	<10
61/F	10	<10	10	<10	<10	10	<10
61/M	10	<10	160	160	<10	<10	320
61/M	<10	<10	<10	<10	<10	<10	<10
61/F	<10	<10	10	<10	<10	<10	<10
62/F	<10	20	10	20	NR	NR	NR
62/F	10	10	80	20	<10	<10	40
62/F	<10	<10	20	10	<10	20	40
62/F	<10	<10	80	40	<10	<10	80
63/M	<10	<10	80	40	<10	<10	80
63/F	<10	<10	10	<10	<10	<10	<10
63/M	<10	<10	40	20	<10	<10	20
63/M	<10	<10	40	80	<10	<10	20
64/F	10	<10	20	10	<10	10	10
65/F	10	<10	10	<10	<10	20	<10
66/M	<10	<10	<10	<10	<10	<10	<10
68/M	<10	<10	40	80	<10	<10	40
71/F	10	10	10	20	<10	<10	<10
72/F	<10	20	10	20	10	10	<10
75/M	<10	<10	10	10	<10	20	<10
76/M	<10	<10	40	20	<10	<10	40
78/F	<10	10	10	20	<10	<10	<10
82/F	10	10	20	10	<10	20	<10

*WHO, World Health Organization; HI, hemagglutination inhibition; MN, microneutralization; NR, no results (because of insufficient serum).
†Tested with guinea pig erythrocytes.
‡Tested with turkey erythrocytes.

Europe (*8*), resident populations in these countries have been exposed to these virus lineages more frequently than populations in Asia, and therefore may have acquired a greater degree of preexisting cross-reactive immunity to pandemic (H1N1) 2009 virus. A recent review of human swine influenza infections suggests that they may not be uncommon (*9*), although the true incidence of human infections with swine influenza is unknown because of paucity of swine influenza surveillance data worldwide (*8*).

In conclusion, partial cross-immunity and cell-mediated immunity may be present but not detected by HI or MN assays. Thus, results of standard serologic assays may not be providing all relevant data (*10*).

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Molecular Epidemiology of Japanese Encephalitis Virus, Taiwan

To the Editor: Japanese encephalitis virus (JEV) is a mosquito-borne member of the family *Flaviviridae* and the genus *Flavivirus*. JEV is a major cause of viral encephalitis in Asia. Phylogenetic analysis of the envelope (E) gene sequences has shown that JEV strains can be clustered into 5 distinct genotypes (*1*). Among them, genotype III (GIII) has had the widest geographic distribution in countries in Asia, including Japan, South Korea, People's Republic of China, Taiwan, Vietnam, the Philippines, and India (*2*). Before 1990, GIII had been the major epidemic JEV type in these areas. However, the introduction of JEV genotype I (GI) has been reported in Japan, Vietnam, South Korea, Thailand, and China in the past decade (*3*–*6*). Nabeshima et al. recently reported surveillance results that provided substantial evidence of frequent introductions of JEV GI into Japan from Southeast Asia and continental eastern Asia (*7*). Because all current vaccines are derived from JEV GIII strains, the effectiveness of vaccination in inducing protective neutralizing antibodies against various genotype strains needs to be carefully evaluated, taking into account genotype shift in these countries.

Japanese encephalitis is endemic in Taiwan. Reports on the molecular epidemiology of JEV in Taiwan are scarce. Jan et al. (*8*) reported the genetic variation of 47 JEV isolates from Taiwan before 1994. Phylogenetic analysis showed that all Taiwanese isolates were GIII, and they were classified into 3 clusters.

To understand the genetic variation of JEV strains currently circulating in Taiwan, we conducted a surveillance program in the following areas: northern (Taipei, Taoyuan, and Yilan

counties and Taipei City), central (Taichung and Changhua counties), southern (Tainan and Kaohsiung counties), and eastern (Hualien County) during 2005–2008. Real-time reverse transcription–PCR (RT-PCR) was used to screen JEV in mosquito pools, pig serum specimens, and human cerebrospinal fluid as described (*9*). Mosquitoes were pooled by species, location, and collection date in groups of 30–50 mosquitoes. Mosquito pools were homogenized and clarified by centrifugation, and the supernatants were sterilized by filtration and removed for real-time RT-PCR and virus isolation.

We used 3 sets of primers for real-time RT-PCR: flavivirus-specific (FL-F1: 5′-GCCATATGG TACATGTG-GCTGGGAGC-3′; FL-R3: 5′-GTKA TTCTTGTGTCCCAWCCGGCTGT GTCATC-3′; FL-R4: 5′-GTGATGCG RGTGTCCCAGCCRGCKGTGT CATC-3′), JEV-specific (*10*) (JE3F1: 5′-CCCTCAGAACCGTCTCGGAA-3′ and JE3R1: 5′-CTATTCCCAGGTG TCAATATGCTGT-3′), and JEV GIII–specific (E12F: 5′-CTGGGAATGG GCAATCGTG-3′ and E325R: 5′-TGTCAATGCTTCCCTTCCC-3′). Samples with positive results by RT-PCR were subjected to virus isolation by using a mosquito C6/36 cell line. A total of 47 JEV isolates were obtained: 38 from mosquitoes, 8 from pig serum samples, and 1 from human cerebrospinal fluid.

Viral RNA was extracted from JEV-infected culture medium, and RT-PCR and DNA sequencing were performed to determine the complete E gene sequences of JEV isolates. Multiple sequence alignment and phylogenetic analysis were conducted by using CLUSTALW software (www.ebi.ac.uk/Tools/clustalw2/index.html) and MEGA version 4 (www.megasoftware.net). The phylogenetic tree was constructed by the neighbor-joining method and the maximum composite likelihood model.

The Figure shows the phylogenetic tree derived from 67 samples of E gene sequences, including 28 representative new sequences in this study (GenBank accession nos. GQ260608–GQ260635), 10 sequences of Taiwanese strains isolated before 2002, and 29 sequences from GenBank. The results show that isolates from Taiwan comprised 2 genotypes, GIII and GI. All of the JEV isolates from Taiwan obtained during 2005–2008, except

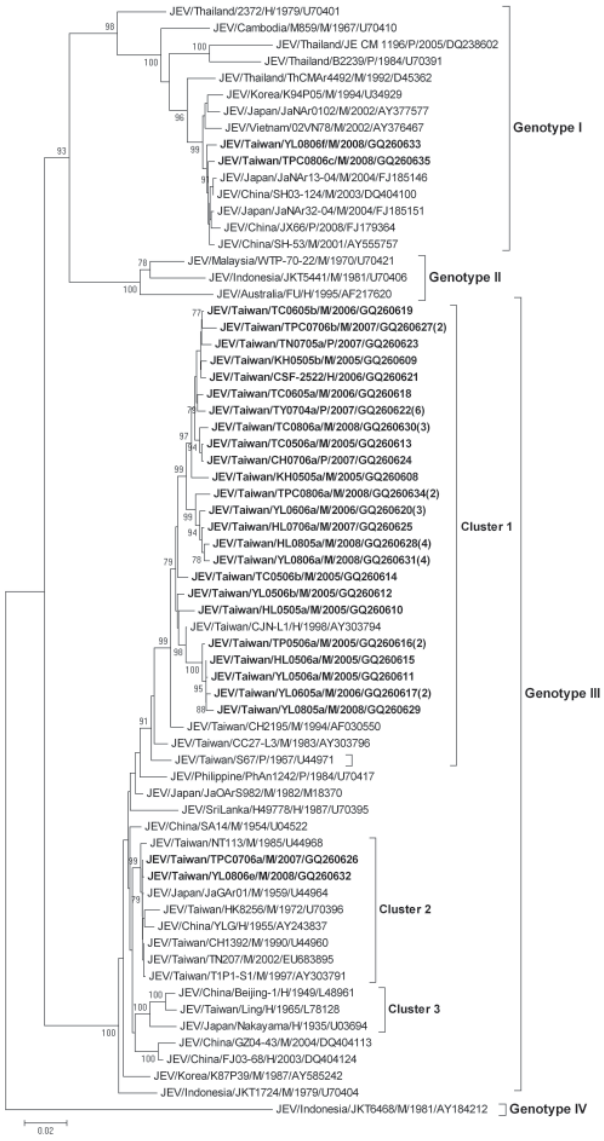

Figure. Phylogenetic tree showing the genetic relationship among Japanese encephalitis virus (JEV) isolates. The tree was constructed on the basis of complete envelope (E) nucleotide sequences of JEV strains. Sequences obtained in this study are indicated in **boldface**. Genotypes are indicated on the right. Viruses were identified by using the nomenclature of virus/country/strain/source/year of isolation/GenBank accession number. Numbers in parentheses indicate the number of isolates that showed 100% nucleotide homology. Isolates with the same sequences were collected at the same time from the same location in this study. Analysis was performed by using MEGA 4 software (www.megasoftware.net) and neighbor-joining (maximum composite likelihood) methods. Bootstrap support values >75 are shown (1,000 replicates). CH, Changhua County; HL, Hualien County; KH, Kaohsiung County; TC, Taichung County; TN, Tainan County; TP, Taipei County; TPC, Taipei City; TY, Taoyuan County; YL, Yilan County; M, mosquito pool; p, pig serum; H, human sample. Scale bar indicates nucleotide substitutions per site.

2 strains (TPC0806c/M/2008 and YL0806f/M/2008), belonged to GIII and formed into 2 clusters.

Cluster 1 contains most new isolates prevalent in different areas of Taiwan. Although cluster 1 isolates are closely related to other JEV strains from Asia, these isolates, together with previously published JEV sequences from Taiwan, form a distinct lineage and may have been continuously evolving and locally adapting in Taiwan. Cluster 2 contains only 2 new isolates, TPC0706a/M/2007 and YL0806e/M/2008, which were isolated from the *Culex tritaeniorhynchus* mosquito pools in Kuantu Nature Park, Taipei City, and from a pig farm in Wujie Township, Yilan County, respectively.

Notably, the 2 GI strains, TPC0806c/M/2008 and YL0806f/M/2008, were isolated from the same areas as the GIII cluster 2 strains. These areas are adjacent to the wetlands, which are stopover sites for migratory birds. These 2 GI strains are most closely related to the strains of the subcluster II JEV strains reported by Nabeshima et al. (*7*). The TPC0806c/M/2008 GI strain is most closely related to Japan/JaNAr13–04/M/2004 and China/SH03–124-/M/2003 strains (99.5% and 99.4% identities, respectively), and the YL0806f/M/2008 GI strain is most closely related to Japan/JaNAr13–04-/M/2004 and China/JX66/P/2008 strains (99.3% and 99.3% identities, respectively). Therefore, JEV GI strains from Taiwan were likely introduced by water birds migrating back and forth along the Asia–Australasia flyway, which passes through many countries, including Indonesia, Malaysia, Australia, the Philippines, Taiwan, China, and Japan (*3*).

Our results clearly showed that JEV GIII strains remain the most dominant population circulating in Taiwan, although 2 JEV GI strains were isolated from wetland areas in northern Taiwan in 2008. Further studies are needed to continuously monitor the changing epidemiologic pattern of JEV strains endemic in Taiwan and newly introduced viruses.

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Fluoroquinolone-Resistant Typhoid, South Africa

To the Editor: *Salmonella enterica* serotype Typhi, the causal pathogen for typhoid, is a major public health hazard in many parts of the world, with an estimated 21.6 million cases of typhoid and 217,000 deaths occurring each year (*1*). Most isolates in South Africa are susceptible to quinolones, and fluoroquinolones remain the treatment of choice (*2*). The disease is primarily water or foodborne, but person-to-person spread is well recognized (*3*). Travelers to disease-endemic regions may be at risk for typhoid, which may result in the importation of strains of *S.* Typhi with unfamiliar or unusual resistance patterns (*4*). Such infections present a challenge to local clinicians on optimal patient management.

S. Typhi was isolated from the blood culture of a woman 65 years of age from Cape Town; she had been in contact with a traveler to Bangladesh. The patient was treated first with ciprofloxacin, but this medication was changed to high-dose ceftriaxone combined with doxycycline for 8 days; she recovered well. Contact tracing indicated no family members had typhoid fever or carried the organism. The person who had traveled to Bangladesh was unavailable to provide further history or a stool specimen. No other potential source of infection could be elucidated: the patient lived in an urban area with safe water sources and shared meals with her family.

The isolate was referred to the Enteric Diseases Reference Unit for confirmation of identification, serotyping (Kauffman-White scheme), and antimicrobial drug susceptibility testing using the Etest (bioMérieux, Marcy l'Étoile, France) and agar dilution methods, according to criteria of the Clinical and Laboratory Standards Institute (Wayne, PA, USA) (www.clsi.org). The isolate was resistant to ampicillin, chloramphenicol, sulfamethoxazole, nalidixic acid, and ciprofloxacin, but susceptible to ceftriaxone and tetracycline.

Pulsed-field gel electrophoresis (PFGE) analysis was performed on the isolate, following the standard PulseNet protocol (*5*). The PFGE pattern was compared with a database of *S.* Typhi PFGE patterns from South Africa by using BioNumerics version 6.01 software (Applied Maths, Sint-Martens-Latem, Belgium). The PFGE pattern of this isolate was 100% identical to pattern JPPX01.0026 in the Global PulseNet *Salmonella* Typhi Database. This pattern, which has been reported to PulseNet from India, Kenya, Tanzania, and Taiwan, is the most common pattern in the database (www.pulsenetinternational.org/projects/styphidatabase.asp); it is rarely seen in South Africa (Figure).

PCR was used to isolate the quinolone resistance-determining region (QRDR) of *gyrA*, *gyrB*, *parC,* and *parE* (*6*). Genes were sequenced by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an Applied Biosystems 3130 genetic analyzer. The QRDR DNA sequences were compared with those of *S.* Typhi strain Ty2 (GenBank accession no. AE014613). PCR also used to confirm the presence of *qnrA*, *qnrB,* and *qnrS* genes (*6*). Analysis for mutations in the QRDR of *gyrA*, *gyrB*, *parC,* and *par* found a single amino-acid mutation (Ser83 to Tyr) in g*yrA*. No amino acid mutations were identified in g*yrB*, *parC*, and *parE*. PCR for detection of *qnr* genes confirmed the presence of a *qnrS* gene, which was identified as the *qnrS1* variant by nucleotide sequence analysis.

The efflux of quinolones from bacterial cells was investigated in the following manner. For nalidixic acid and ciprofloxacin, agar dilution MIC testing was performed in the absence and presence of 40 μg/mL of the efflux pump inhibitor, Phe-Arg-β-naphthylamide (*6*). In the presence of efflux pump inhibitor, the MIC to ciprofloxacin decreased from 4 μg/mL to 1 μg/mL, and the MIC to nalidixic acid decreased from >512 μg/mL to 32 μg/mL, establishing the involvement of an efflux pump in conferring quinolone resistance.

The mutation in g*yrA* (Ser83 to Tyr) confers reduced susceptibility to ciprofloxacin to a maximum MIC ≈0.5 μg/mL (*7*) and the QnrS1 protein confers reduced susceptibility to ciprofloxacin to a maximum MIC ≈0.5 μg/mL (*8*). We showed that a single amino acid mutation in *gyrA* (Ser83 to Tyr) with the QnrS1 protein and active efflux, conferred ciprofloxacin resistance, at least to an MIC level of 4 μg/mL. Previously, Smith et al. reported

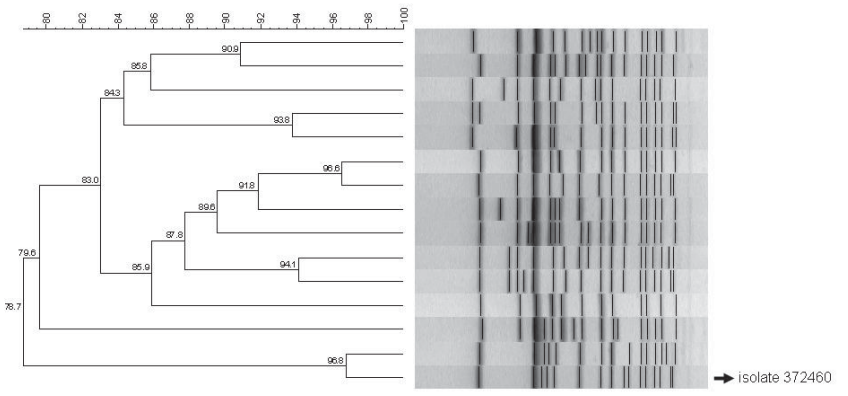

Figure. Dendrogram of pulsed-field gel electrophoresis patterns representative of the 15 largest clusters of *Salmonella enterica* serotype Typhi isolates identified in South Africa during 2005–2009. The pattern of isolate 372460 is indicated. Scale bar represents percentage similarity of pathogens.

quinolone resistance in South African isolates of *S.* Typhi mediated by mutations in *gyrA* and *parC* in combination with active efflux (*6*). We report *qnrS1* from *S.* Typhi, confirming the role of plasmid-mediated fluoroquinolone resistance in *S.* Typhi (*9*) and a fluoroquinolone-resistant strain in South Africa. Fluoroquinolone resistance is well recognized in Bangladesh; other researchers have described multidrug-resistant *S.* Typhi isolates imported from that country (*10*). Molecular epidemiology supports the conclusion that this strain likely originated in Bangladesh (L. Theobald, pers.comm.).

In conclusion, fluoroquinolone-resistant typhoid fever is a reality in South Africa in patients who have a history of travel or contact with travelers. Blood cultures are mandatory to guide antimicrobial drug management. Plasmid-mediated fluoroquinolone resistance has implications for cotransference of resistance to the major antimicrobial agents used to treat typhoid fever and for the potential for rapid spread of fluoroquinolone resistance through *S.* Typhi strains in South Africa. The presence of fluoroquinolone-resistant typhoid fever could force a change in current treatment guidelines for this disease.

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Sorbitol-fermenting *Escherichia coli* O157, Scotland

To the Editor: Verotoxin-producing *Escherichia coli* (VTEC) of serogroup O157 causes severe gastrointestinal and renal illness; clinical signs may be mild diarrhea, hemorrhagic colitis, or hemolytic uremic syndrome (HUS). Typically, 10%–15% of reported VTEC infections quickly progress to HUS (*1*). Sorbitol-fermenting (SF)–O157 strains have emerged in continental Europe (*2*,*3*). Some evidence suggests that SF-O157 is more frequently associated with HUS than are non-sorbitol–fermenting strains (*3*–*6*). SF-O157 shows increased adherence to colonic epithelial cells and may in turn cause a more potent inflammatory host response, resulting in a higher risk for HUS (*4*). The potentially greater virulence of SF-O157 requires urgent identification of its reservoir(s) and vehicle(s) of infection, as well as determination of genetic or other predisposing factors for infection with this strain. To understand whether the host pathophysiologic responses to SF-O157 and non–SF-O157 strains differ, we analyzed a cohort of children with HUS who were infected with *E. coli* O157.

During April and May 2006, Health Protection Scotland (HPS) identified 18 cases of verotoxin-producing SF-O157 infection in Scotland, 13 of which were associated with a nursery. HUS developed in 8 of the 18 patients; those with thrombotic microangiopathy were admitted to the renal unit of a specialist pediatric hospital, which immediately reports cases of HUS to HPS as part of national surveillance (*7*). To test the hypothesis that SF-O157 was more virulent than non–SF-O157, we performed an age-matched, nested case–case study of HUS case-patients and analyzed host clinical markers, treatment, and outcomes from SF-O157 and non–SF-O157 cases in 2006. Clinical questionnaires, patient information sheets, and consent forms were completed by clinicians for each case-patient and returned to HPS; data were entered into a database in Epi Info version 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Statistical analysis by *t* test showed that nadirs for serum albumin were significantly higher for children with SF-O157 HUS ($p = 0.03$; Table) than for children with non–SF-O157 HUS and that children with SF-O157 HUS had significantly more sessions of hemodialysis than did children with non–SF-O157 HUS ($p = 0.01$; Table). All case-patients were oligoanuric; the 2 groups did not differ with respect to this parameter. Initial signs and symptoms were similar for both sets of patients, i.e., classic VTEC symptoms of bloody diarrhea and abdominal pain. This finding is in acccordance with those of other studies of SF-O157 outbreaks, which also noted signs and symptoms compatible with VTEC-associated gastroenteritis (*5*,*6*).

Our study highlights a number of lessons. Medical practitioners rarely have the opportunity to recognize patients at such an appreciable and predictable risk of progressing rapidly to anuric renal failure as they do when they see children with early O157 infection. Failure to appreciate the potential gravity of O157 infection and the possible development of HUS may result in avoidable illness and even death. Our investigation of the prehospital management of SF-O157 and non–SF-O157 in this cohort found no difference in pharmacologic intervention or duration of delay in admission to hospital.

Our study has limitations. A number of patients in the cohort were prescribed antimicrobial drugs and/or antimotility drugs or were sent home from the local hospital without hospital admission or further monitoring; such actions potentially exacerbate clinical outcomes (*1*,*8*). We recognize that comparison of the SF-O157 outbreak strain with non–SF-O157 strains (some of which caused sporadic cases) may be a potential confound-

Table. Characteristics of patients infected with non–SF-O157 versus SF-O157 *Escherichia coli*, Scotland, 2006*

Characteristic	SF-O157, n = 8	Non–SF-O157, n = 19	p value
Age, y ± SEM	5.4 ± 1.4	5.1 ± 0.9	
Sign or symptom, no. (%) patients			
Diarrhea	8	19	
Bloody diarrhea	6 (75)	14 (74)	0.79
Abdominal pain	6 (75)	13 (68)	0.13
Fever	1 (12)	4 (21)	0.73
Neurologic involvement	2 (25)	4 (21)	0.82
Clinical parameter, mean ± SEM			
Anuria, d	11.7 ± 2.7	7.9 ± 1.4	0.20
Leukocyte count, $\times 10^9$/L	26.4 ± 2.1	36.4 ± 10.1	0.34
C-reactive protein, mg/L	65.6 ± 27.1	93.4 ± 23.1	0.31
Serum albumin, g/L	32.4 ± 7.0	23.2 ± 1.0	0.03
Lactate dehydrogenase, IU/L	2,774 ± 280	2,556 ± 324	0.68
Hospital stay, d	17.9 ± 3.7	16.1 ± 2.9	0.71
Treatment, mean no. sessions ± SEM			
Peritoneal dialysis	13.4 ± 2.3	7.4 ± 1.9	0.07
Hemodialysis	20.5 ± 3.5	9.3 ± 1.3	0.01
Outcomes, 1 y follow-up, no.	n = 6	n = 19	
Full recovery, no. patients	6	17	
Clinical sequelae, no. patients	0	2†	

*SF, sorbitol-fermenting.
†1 with hypertension, 1 with abdominal pain/vomiting.

ing factor in the analysis. However, recently published work has indicated no statistically significant differences in the verotoxin proteins encoded by SF-O157 or non–SF-O157 strains or in their level of toxicity (*9*). Other virulence factors may contribute to increased likelihood of HUS (*4*).

Our data suggest that infection with SF-O157 results in less severe colitis than does the more common non–SF-O157 infection. Less severe colitis could result in a lower risk for renal disease because less verotoxin would be translocated into the bloodstream and bound to the kidneys. However, patients infected with SF-O157 had anuria for longer periods and consequently had longer sessions of peritoneal and hemodialysis. Although unknown bacterial or host inflammatory cytokines may contribute to enhanced disease progression, this observation is surprising and requires further investigation. Additional research is needed to learn more about the virulence of SF-O157 strains and establish other host factors that contribute to disease progression.

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Co-infection with Dengue Virus and Pandemic (H1N1) 2009 Virus

To the Editor: Dengue is a mosquito-borne viral infection caused by 4 related dengue viruses. Each of these viruses is capable of causing classic dengue fever or dengue hemorrhagic fever (DHF), but may also cause nonspecific febrile illnesses. As a result, dengue is often difficult to diagnose clinically, especially because peak dengue season often coincides with that of other common febrile illnesses in tropical regions (*1*). Concurrent outbreaks of influenza and dengue have been reported (*2*,*3*); this circumstance often leads to delayed recognition of the presence of one or other disease in the community.

In April 2009, a new strain of influenza A virus known as pandemic (H1N1) 2009 virus was first detected in the United States (*4*). Pandemic (H1N1) 2009 infections were first detected in Puerto Rico in June 2009, and 59 deaths caused by the virus have been confirmed to date. This influenza outbreak coincided with the typical dengue season in Puerto Rico, which led to diagnostic difficulties; both infections disproportionately affected young persons, who often had similar, nonspecific symptoms.

We describe a case of laboratory-confirmed co-infection of dengue virus and pandemic (H1N1) 2009, and discuss the difficulties in distinguishing the 2 illnesses clinically. A 33-year-old woman (healthcare worker) in Ponce, Puerto Rico, sought treatment at an emergency department of a hospital in the southern part of the island with a 3-day history of febrile illness. Her symptoms began with throat irritation and earache; subsequently, cough, fever, and headache developed. She reported palpitations and generalized malaise but no other symptoms. The patient had no notable medical his-

tory and denied taking any medicines apart from over-the-counter antipyretics. She reported recent exposure to influenza at work and multiple recent mosquito bites. On physical examination, she had a temperature of 37°C, a heart rate of 91 bpm, and blood pressure of 125/82 mm Hg. A tourniquet test result was positive. Her pharynx was erythematous without exudate, and she had rhinorrhea. She had no lymphadenopathy, rash, petechiae, or purpura. Several small, red papules, which the patient described as recent mosquito bites, were on her legs. The remainder of her examination showed no unusual findings.

Laboratory studies showed a leukocyte count of 5,300 cells/mm^3 with a normal differential count, hematocrit 35.2%, and thrombocyte count of 239,000 cells/dL. Results of a chest radiograph was unremarkable. A nasopharyngeal swab was positive for influenza A virus by rapid test. Nasopharyngeal and serum samples were sent to the Centers for Disease Control and Prevention (Dengue Branch) for influenza and dengue testing. The patient was diagnosed with suspected pandemic (H1N1) 2009 infection and prescribed oseltamivir for 5 days. She returned for a follow-up visit 12 days after the onset of symptoms. She reported having 2 more days of fever after her initial visit, but had no rash, petechiae, bleeding, or progression of respiratory symptoms. A second serum specimen was obtained during this visit.

The initial serum specimen was positive for dengue virus by serotype-specific, singleplex, real-time reverse transcription–PCR (*5*). Her nasopharyngeal specimen was positive by PCR for pandemic (H1N1) 2009 influenza. The second, convalescent-phase, serum specimen was negative for dengue immunoglobulin (Ig) M by IgM antibody–capture ELISA. The acute-phase and convalescent-phase samples were positive for IgG against dengue by ELISA (*6*), which indicated a secondary dengue infection. IgG titers exceeded the limits of the test for acute-phase and convalescent-phase samples, showing unusually elevated levels of IgG against dengue.

Distinguishing dengue and influenza by clinical features alone can be difficult. In an investigation of simultaneous dengue and influenza A outbreaks in Puerto Rico in 1977, similar percentages of persons with confirmed dengue and confirmed influenza had classic dengue symptoms (*2*). Hemorrhagic manifestations, like those typically seen in DHF, have been reported with influenza A in prior outbreaks (*7*,*8*) and with pandemic (H1N1) 2009 (Centers for Disease Control and Prevention, unpub. data). Previous influenza A outbreaks were initially believed to be outbreaks of DHF until careful laboratory investigation proved otherwise (*8*). Our patient did not have the typical signs and symptoms of dengue (rash, eye pain, thrombocytopenia, arthralgia, petechiae, or bleeding) that would differentiate her condition from that of patients with other febrile illnesses. She did have a positive tourniquet test result and fever, which have been advocated as screening criteria for dengue infection in children, at the time of initial examination (*9*). Data for the specificity and sensitivity of these criteria in adults are sparse, however, and some studies have shown a high incidence of positive tourniquet test results in patients with laboratory-confirmed influenza (*7*,*8*).

Our report demonstrates that co-infection with dengue virus and pandemic (H1N1) 2009 can occur. Previous studies also have shown cases of probable co-infection with seasonal influenza and dengue (*1*,*10*), including 1 fatal case (*1*). Because many dengue-endemic countries are experiencing pandemic (H1N1) 2009 outbreaks, providers should consider the possibility of viral co-infection, especially in severe cases, and should consider testing for both viruses.

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Bovine Tuberculosis in Buffaloes, Southern Africa

To the Editor: Emergence of bovine tuberculosis (TB) in wildlife in southern Africa has implications not only for the conservation of the wildlife species affected (*1*) but also for the health of humans and livestock living at the wildlife–livestock–human interface (*2*). Bovine TB in South Africa's Kruger National Park was first found in African buffaloes (*Syncerus caffer*) in 1990 (*3*) and likely entered the park by cattle-to-buffalo transmission (*4*). Bovine TB infection has been spreading northward; in 2003, infection was confirmed in a buffalo ≈60 km south of the Limpopo River. In 2005, a case was confirmed only 6 km south of the river (D. Keet, unpub. data). In 2008, we isolated *Mycobacterium bovis* from African buffaloes in Zimbabwe.

During October 9–13, 2008, a total of 38 buffaloes from 4 herds were captured in Gonarezhou National Park (south of the Mabalauta area; 22.0553°S, 31.4265°E). Blood samples were collected, sampled buffaloes were marked and released, and 3 adult females in each herd were equipped with radio collars. Buffalo tissue samples were collected, packaged, shipped, and handled at the Agricultural Research Council–Onderstepoort Veterinary Institute according to procedures recommended for controlling the spread of foot-and-mouth disease virus. Interferon-γ assay (*5*) results were positive for bovine TB for 4 (10.5%) buffaloes: 2 adult females and 1 young adult male from the same herd and 1 adult female from another herd.

Four months later, a radio-collared adult female and the young adult male, each of which had had positive interferon-γ assay results, were sedated and euthanized, and necropsies were performed in the field. Samples for histopathologic examination and culture were collected from lymph nodes of the head and thorax. No acid-fast organisms were detected, but the histologic findings were strongly suggestive of paucibacillary TB. *M. bovis* was isolated from the retropharyngeal lymph nodes of both buffaloes and from the bronchial and head lymph nodes of 1 of them. Both isolates were typed by analysis of variable number of tandem repeat (VNTR) sequences at 6 loci (exact tandem repeat A–F) (*6*) and compared with the VNTR profiles of ≈75 isolates from Kruger. All isolates showed an identical VNTR profile (7544*52.3), which suggests an epidemiologic link between the *M. bovis* infections in the 2 parks. However, the exact tandem repeat loci had lower discriminatory power among Kruger isolates than did IS*6110* restriction fragment length polymorphism typing (T. Hlokwe, unpub. data) (*4*). A typing regimen comprising different typing methods and markers will be useful for more accurately determining the genetic relationship between the isolates from the 2 parks, Gonarezhou and Kruger.

The confirmation of results for bovine TB–infected buffaloes in Zimbabwe (Gonarezhou National Park) raises several questions regarding the transboundary spread of animal disease and has considerable management implications for the Great Limpopo Transfrontier Conservation Area. The most likely scenario is buffalo-to-buffalo contact across the boundary because the bovine TB cases reported here were located <45 km from the unfenced northern boundary of Kruger National Park. Buffaloes, especially bulls and young heifers, frequently move from herd to herd and may contribute to the spread of *M. bovis* by mixing with unexposed herds (*7*). Although transboundary movements of buffaloes between Kruger and Gonarezhou have not been specifically documented, uncontrolled movements across the Limpopo River do occur (de Garine-Wichatitsky, unpub. data). However, >12 wild species in Kruger have now been found to be infected by bovine TB (*2*). Most of these species are probably not effective sources of *M. bovis* infection for buffaloes, but the disease epidemiology could rely on multihost reservoirs (*8*). Thus, a second scenario could be a buffalo-to–unidentified wild species–to-buffalo pathway, because species like greater kudu (*Tragelaphus strepsiceros*) appear to be able to maintain, spread, and even drive a bovine TB epidemic (*4*,*9*). A third scenario involves movement of infected livestock across the boundaries of the 3 countries of the Great Limpopo Transfrontier Conservation Area, resulting in cattle-to-buffalo transmission of bovine TB. As a last scenario, we cannot rule out the possibility that bovine TB infection of buffaloes has remained silent and undetected for decades in Zimbabwe.

The management implications of bovine TB in buffaloes in Gonarezhou National Park are considerable. Once bovine TB is established in a native free-ranging maintenance host, eradication is unlikely (*2*,*10*). Evaluation

of the prevalence and distribution of the infection in wildlife and livestock populations on the Zimbabwe side of the Great Limpopo Transfrontier Conservation Area is urgently needed. Control options in wildlife are limited (*2*,*10*), but chances of success are greater if control measures are initiated at the early stage of disease spread into a new area. Adequate risk-mitigation strategies should be developed and implemented to reduce the risk for bovine TB transmission to livestock and humans living at the periphery of the unfenced Gonarezhou National Park. Failure to promptly assess the situation and adopt appropriate measures would have far-reaching conservation, economic, and public health consequences, not only for Zimbabwe but also for the political and social acceptance of the transfrontier conservation areas in southern Africa.

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No Resistance Plasmid in *Yersinia pestis*, North America

To the Editor: Plague, caused by *Yersinia pestis*, is now largely controlled by improved sanitation and the use of antimicrobial drugs. However, before the widespread availability of antimicrobial drugs, an estimated >200 million persons died during pandemics (*1*). Today, if *Y. pestis* were to acquire antimicrobial drug resistance determinants, plague could again be a deadly disease.

Antimicrobial drug resistance in *Y. pestis* has been documented for

only a few strains. The best available information is for 2 strains isolated in Madagascar in 1995 (*2*), in which resistance was conferred by plasmids not typically found in *Y. pestis*. Strain 16/95 was resistant to streptomycin only; this resistance was mediated by plasmid pIP1203 (*3*). Strain 17/95 was resistant to 8 antimicrobial drugs, including some commonly used to treat plague, such as streptomycin, tetracyclines, and sulfonamides (*2*). Multidrug resistance in 17/95 was mediated by plasmid pIP1202 (*4*). Both plasmids could be transferred by conjugation from the source *Y. pestis* strains to other *Y. pestis* strains and *Escherichia coli* (*3*,*4*). pIP1203 could be transferred from *E. coli* to *Y. pestis* in the midgut of co-infected rat fleas (*Xenopsylla cheopis*), common vectors of plague (*5*).

Comparative sequence analysis has indicated that pIP1202 shares an almost identical IncA/C backbone with multidrug-resistant (MDR) plasmids from *Salmonella enterica* serotype Newport SL254 and *Yersinia ruckeri* YR71, suggesting recent acquisition from a common ancestor (*6*). In this study, this backbone was detected in numerous MDR enterobacterial pathogens (e.g., *E. coli*, *Klebsiella* spp., and multiple *Salmonella* serotypes) isolated from retail meat products. Many of these plasmids transferred at high rates to a plasmid-free *Y. ruckeri* strain, indicating the ability to efficiently transfer among species. Meat products examined in that study originated from 9 US states, including western plague-endemic states such as California, Colorado, New Mexico, and Oregon.

To determine whether the IncA/C plasmid backbone previously found in MDR *Y. pestis* and other species exists in *Y. pestis* isolates from western U.S. states, we screened *Y. pestis* DNA. The 713 isolates were collected from from humans, small mammals, and fleas in 14 of the 17 western plague-endemic states (Table), including all states that reported human cases during 1970–2002 (*7*). We used Primer Express software (Applied Biosystems, Foster City, CA, USA) to design a TaqMan-MGB single-probe assay to detect *repA*, a plasmid replication gene present in the IncA/C plasmid backbone. We based this assay on the *repA* assay described by Carrattoli et al. (*8*) and used the same forward primer but a different reverse primer and an additional probe to facilitate screening on a real-time PCR platform.

Real-time PCRs were conducted in 10-μL reaction mixtures that contained 900 nmol/L of forward (5′-GAGAACCAAAGACAAAGACCTGGA-3′) and reverse (5′-TGGCCGGAGATTCAATGATC-3′) primers, 200 nmol/L of the *repA*-specific probe (5′-6FAM-AGACTCACCGCAAATG-3′), 1× AB TaqMan Universal PCR Master Mix with AmpErase UNG (uracil N-glycosylase) (Applied Biosytems), and 1 μL of template. Thermal cycling was performed on an Applied Biosystems 7900 HT sequence detection system under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. DNA extracts from *Y. pestis* strain 17/95 (*4*) and *Salmonella enterica* serotype Newport strain SL254 (*6*) were used as positive controls.

Of the 713 *Y. pestis* isolates screened, none was positive for the IncA/C plasmid backbone, indicating that MDR as mediated by pIP1202-like MDR plasmids described by Welch et al. (*6*) was not in these samples. This finding is encouraging with regard to public health. However, we screened only for the plasmid backbone; MDR genes may have been in some of these samples but not carried by pIP1202-like MDR plasmids, especially considering that plasmids can be readily integrated into the *Y. pestis* chromosome (*1*).

Could MDR *Y. pestis* arise in North America by acquisition of an MDR plasmid, such as pIP1202, from food-animal production activities in plague-endemic regions? If so, *Salmonella* spp. would be a likely MDR plasmid donor for several reasons. First, *Y. pestis* has several plasmids that are highly similar to those in *Salmonella* spp., indicating active transfer of plasmids between these 2 bacterial groups (*6*). Second, fleas that are common vectors of plague have been shown to be naturally co-infected with *Salmonella* spp. and *Y. pestis* and capable of transmitting both organisms to rodent hosts (*9*). Third, MDR plasmids are readily transferred to *Y. pestis* in the flea gut (*5*). Fourth, transferable MDR plasmids are common among *Salmonella*

Table. *Yersinia pestis* isolates collected from humans, small mammals, and fleas, United States

State	No. isolates	Years collected
Arizona	151	1975, 1977–1984, 1986–1989, 1992–1996, 1998, 2000–2002
California	129	1943, 1962, 1970, 1977, 1979–1980, 1983–1999
Colorado	97	1963, 1968, 1989, 1992, 1995–1997, 1999–2002
Idaho	2	1987, 1997
Kansas	17	1997, 1999
Montana	11	1987, 1992–1993
North Dakota	2	1986, 1993
New Mexico	124	1950, 1976–1977, 1979–1988, 1991–1992, 1994–1995, 1997–2002
Nevada	36	1980–1985, 1987, 1992–1995
Oregon	18	1959, 1970–1971, 1977, 1979, 1981–1984, 1987
Texas	5	Unknown
Utah	55	1965, 1981–1984, 1991–1995, 1999–2001
Washington	2	1984
Wyoming	64	1978, 1980, 1982–1983, 1985–1987, 1989–1990, 1997, 2000–2001

spp. isolates in US food animals (*10*). Given these linkages, the transfer of an MDR plasmid from *Salmonella* spp. to *Y. pestis* seems possible. However, we emphasize that to date no evidence supports this type of event.

The Centers for Disease Control and Prevention provided many of the DNA samples.

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Triatoma infestans Bugs in Southern Patagonia, Argentina

To the Editor: *Triatoma infestans* bugs, the main vector of Chagas disease, historically occupied a large area from northeastern Brazil to Chubut Province in Patagonia, Argentina (*1*). Large-scale insecticide spraying during the 1980s and 1990s reduced its geographic range and abundance and interrupted transmission of *Trypanosoma cruzi*, mainly in Uruguay, Chile, and Brazil (*2*). However, *T. infestans* and transmission of *T.* cruzi persist in the Gran Chaco, a large ecoregion in Argentina, Bolivia, and Paraguay (*3*).

Chubut Province has historically been an area with no risk for vector-mediated transmission of *T. cruzi*; only its extreme northern region was categorized as having a low transmission risk (*4*,*5*). However, increased immigration from disease-endemic rural areas in Argentina and Bolivia into Chubut has raised concerns about accidental introduction of *T. infestans* in travelers' luggage (*1*) and establishment of a transmission cycle.

In January 2007, we conducted a province-wide survey of 21 villages in Chubut Province previously infested with *T. infestans* bugs by using 0.2% tetramethrin as a dislodgant agent (1 person-hour/house); no *T. infestans* bugs were detected (online Appendix Figure, www.cdc.gov/EID/content/16/4/887-appF.htm). Only *T. patagonica* bugs were found in 11% of peridomestic structures, and none were infected with *T. cruzi*. In June 2007, a *T. infestans*–like bug was found in a primary healthcare center in Comodoro Rivadavia (45°51′S, 67°28′W), a city in southern Chubut Province (online Appendix Figure). Healthcare center staff reported visits by immigrants from Bolivia a few days before this finding and suspected them to be the source. The bug was identi-

fied morphologically as a *T. infestans* female and it laid 6 eggs. PCR amplification of kinetoplast DNA showed that it was not infected by *T. cruzi*.

DNA sequence analysis is useful for investigating evolutionary history and population structure within Triatominae (*6*). *T. infestans* bugs from Bolivia and Argentina showed genetic differences for nuclear (*7*) and mitochondrial markers (*6*), including mitochondrial cytochrome oxidase I (mt*COI*) (*8*). We used our mt*COI* haplotype database, which includes published (*8*) and new domestic, peridomestic, and sylvatic *T. infestans* from 65 locations in 13 provinces in Argentina (n = 346) and 3 departments in Bolivia (n = 144), to analyze the mt*COI* sequence of the bug found in southern Patagonia and determine if it could be assigned to a known haplotype from Bolivia or Argentina. We investigated phylogenetic relationships with other haplotypes by using neighbor-joining and Bayesian approaches.

Our mt*COI* database included 53 haplotypes: 42 were found in Argentina, 9 in Bolivia, and 2 in both countries (Figure). The bug from southern Patagonia had haplotype *x*, which has been found in only 3 western or southern provinces in Argentina (San Juan, San Luis, and Rio Negro) (*8*; online Appendix Figure).

Results of phylogenetic analyses were congruent (Figure). The neighbor-joining tree showed that haplotype *x* formed a cluster with haplotype *h* (Argentina) and haplotypes from Bolivia clustered in 3 other groups: 1) two groups with bootstrap values >70% (one with haplotypes *at*, *n*, *c*, and 33 haplotypes from Argentina, and the other with haplotypes *ab*, *ac*, *ad*, *ae*, *ap*, and *az*); and 2) one group with a bootstrap value of 68% (haplotypes *ax* and *aa*). The Bayesian tree showed that haplotypes from Bolivia were arranged in 2 well-supported clades (posterior probabilities ≥83%) and that haplotype *x* was not included within any of them. Thus, haplotype *x* of the bug from southern Patagonia was found only in Argentina and was not closely related to haplotypes from Bolivia.

We investigated the geographic origin of non-native putative attendees of the healthcare center in San Cayetano. These persons were immigrants from Bolivia and from northern (Salta and Jujuy), western (Mendoza, San Juan, and San Luis), and southern Argentina (Rio Negro), i.e., from the 3 putative sources of the bug. These immigrants typically pay extended visits to their home towns at least once a year and transport luggage in which the bug could have traveled. In 2006, San Juan had the highest levels of domestic and

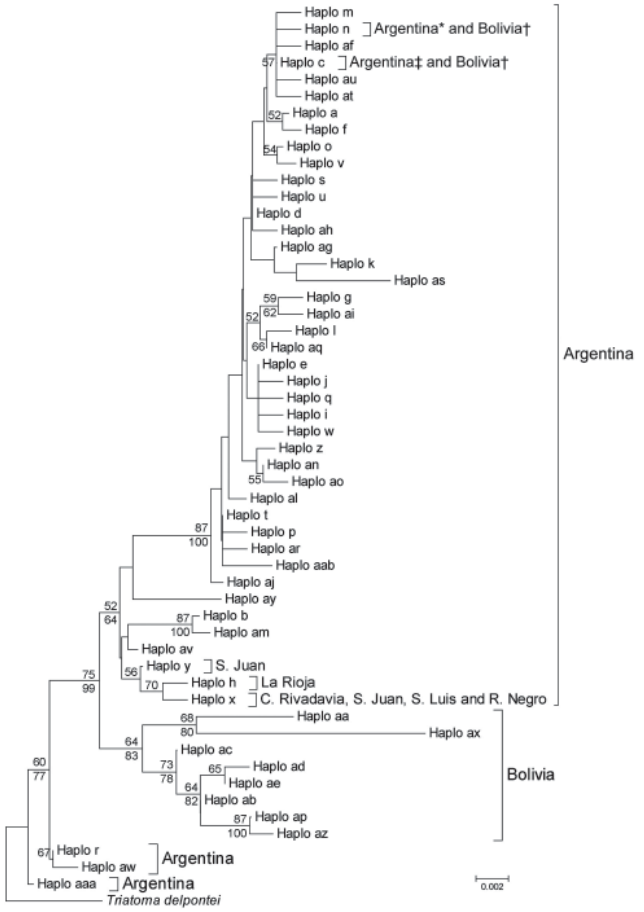

Figure. Phylogenetic relationships between mitochondrial cytochrome oxidase I gene haplotypes of *Triatoma infestans* from Argentina and Bolivia. The neighbor-joining tree was constructed by using MEGA 4.1 (www.megasoftware.net) and bootstrap values (based on 1,000 replications) >50% are shown above the branches. A Bayesian maximum clade credibility tree was similar, and clade posterior probabilities >50% are shown below the branches of the neighbor-joining tree. MRBAYES 3.1 (http://mrbayes.csit.fsu.edu) default priors were assumed and run for 4 million generations. Convergence of the Markov chain Monte Carlo analysis was investigated with the SD of split frequencies and diagnostics implemented in AWTY (http://ceb.csit.fsu.edu/awty). The model of evolution (Hasegawa-Kishino-Yano + invariable sites+ Γ [HKY + I + Γ]) was chosen with Mrmodeltest 2.3 (www.abc.se/~nylander). Because MEGA 4.1 does not support HKY; the more inclusive Tamura-Nei method (www.megasoftware.net/WebHelp/part_iv___evolutionary_analysis/computing_evolutionary_distances/distance_models/nucleotide_substitution_models/hc_tamura_nei_distance.htm) was used for the neighbor-joining analysis. Haplotypes *al*, *an*, *ao*, *ap*, *aq*, *at*, *au*, *ax*, *az*, *aaa*, and *aab* are reported. DNA sequences are available in GenBank (accession nos. EF451005-EF451041, FJ439768, FJ811845–8, and GQ 478993–GQ 479005). *Two provinces in Argentina; †Tarija, Bolivia; ‡10 provinces in Argentina. Scale bar indicates nucleotide substitutions per site.

peridomestic infestation with *T. infestans* (35% and 21%, respectively), including urban infestation (*9*). Mendoza (not in our database) had considerable domestic and peridomestic infestations (both 7%), and San Luis (0.5% and 5.3%, respectively) and Rio Negro (both <0.1%) had low infestations in 2001 (*4*) and thereafter (C. Spillmann, unpub. data). Bolivia, Salta, and Jujuy are excluded as potential sources of the bug because haplotypes closely related to haplotype *x* were not found in these places. Active dispersal from a local source can be ruled out because there is no precedent for *T. infestans* in Comodoro Rivadavia, and the mean temperature in June (8°C) is below the known threshold for flight initiation (23°C) (*10*).

Our results show that molecular phylogenetics can identify passive transport of insects into areas where a disease is not endemic and rule out putative sources supported only by circumstantial evidence. Our findings reinforce the need for sustained and coordinated vector surveillance and control at a regional level (*3*).

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Serologic Survey of Hantavirus Infection, Brazilian Amazon

To the Editor: Since the etiology of hantavirus cardiopulmonary syndrome (HCPS) was recognized in 1993 in the United States (*1*), various hantaviruses have been associated with the syndrome in South America (*2*,*3*). Depending on the viral genotype involved, hantavirus infection can take a wide variety of forms, from asymptomatic or oligosymptomatic to the classic clinical form (*4*,*5*).

The first cases of HCPS in Brazil were reported in the state of São Paulo in 1993 (*6*,*7*). In 2000, an outbreak of HCPS was reported in the municipality of Anajatuba in the state of Maranhão in the Maranhão western

lowlands, a microregion in the Brazilian Amazon (*8*,*9*). An ecologic study in this region identified antibodies against hantaviruses in wild rodents (*Oligoryzomys fornesi* and *Holochilus sciureus*). Analysis of RNA from the viruses isolated from these 2 rodent species showed 2 new hantaviruses, which were named Anajatuba and Rio Mearim, respectively (*10*).

The Maranhão western lowlands is a swampy region that consists of 21 municipalities and ≈400,000 inhabitants. A cross-sectional study was conducted from August 2004 through September 2006 to identify exposures and activities associated with hantavirus infection. A convenience sample comprising 6 of the 21 municipalities in the region was selected: Pinheiro in the north, Vitória do Mearim in the south, São Bento and São João Batista in the east, and Penalva and Viana in the west. In each municipality, a village in the countryside was randomly chosen. All members of those communities were invited to participate. Persons who agreed to take part in the study were interviewed, and a standardized questionnaire was used to collect demographic information. Participants provided blood samples after giving written informed consent. ELISA to detect immunoglobulin (Ig) G antibodies against hantaviruses was performed in the Arbovirology and Hemorrhagic Fevers Department at the Evandro Chagas Institute (Belém, PA, Brazil) by using antigen from the Sin Nombre virus supplied by the Centers for Disease Control and Prevention (Atlanta, GA, USA). Samples were initially screened at 1:100, and all positive results were confirmed in a serial dilution (starting at 1:400) test. Positive samples were those with titers ≥400 (*10*).

Odds ratios (ORs) were estimated by logistic regression. Variables with p≤0.20 in the univariate analysis were subjected to multivariate analysis.

Our study comprised 1,386 persons. The seroprevalence of antihantavirus antibodies in the 6 municipalities was 4.7% (65/1,389), distributed as follows: São João Batista, 2.6% (8/307); São Bento, 3.1% (6/195); Penalva, 3.5% (5/145); Pinheiro, 4.8% (13/273); Viana, 5.6% (9/160); and Vitória do Mearim, 7.8% (24/309). Most persons interviewed were farmers and illiterate and lived in rammed-earth huts. Killing rats in the fields was reported by 24.3% (337/1,389). According to univariate analysis, seeing rats in the dwelling or in the field, having killed rats in the dwelling or in the fields, being a farmer, being male, and being ≥20 years of age were associated with IgG against hantaviruses. No association was found between storage of grain inside a dwelling or presence of natural predators of rodents, e.g., domestic cats and snakes such as the red-tailed boa, and antibodies against hantaviruses (Table).

Because of colinearity between rat exposure variables, only "having killed rats in the field" was submitted to adjusted analysis. In the multivariate analysis, being ≥20 years of

Table. Univariate analysis of risk factors associated with hantavirus infection in humans, Maranhão western lowlands, Brazil, 2006*

Possible risk factor	SNV IgG positive, no. (%), n = 65	SNV IgG negative, no. (%), n = 1,324	OR (95% CI)	p value
Storing grain in the house	38 (58.5)	774 (58.5)	1.00 (0.60–1.66)	0.999
Sweeping the house	53 (81.5)	1176 (88.8)	0.56 (0.29–1.06)	0.076
Seeing rats in the dwelling	47 (72.3)	742 (56.0)	2.05 (1.18–3.56)	0.011
Seeing rats in the fields	41 (63.1)	618 (46.7)	1.95 (1.17–3.27)	0.011
Having killed rats in the dwelling	46 (70.8)	686 (51.8)	2.25 (1.31–3.88)	0.004
Having killed rats in the fields	29 (44.6)	308 (23.3)	2.66 (1.60–4.40)	<0.001
Being bitten by a rat	5 (7.7)	46 (3.5)	2.32 (0.89–6.04)	0.086
Seeing rat feces in the dwelling	34 (52.3)	609 (46.0)	1.29 (0.78–2.12)	0.320
Being a farmer	40 (61.5)	467 (35.3)	2.94 (1.76–4.90)	<0.001
Using rat meat as fishing bait	1 (1.5)	13 (1.0)	1.58 (0.20–12.2)	0.664
Seeing buffaloes in the fields	26 (40.0)	518 (39.1)	1.04 (0.62–1.72)	0.888
Keeping a snake in the house	0	27 (2.0)	Not calculated	0.245
Keeping a cat in the house	33 (50.8)	689 (52.0)	0.95 (0.58–1.56)	0.841
Keeping a dog in the house	39 (60.0)	890 (67.2)	0.73 (0.44–1.22)	0.229
Living in a dwelling close to the fields	47 (72.3)	868 (65.6)	1.37 (0.79–2.39)	0.264
Living near fruit trees	40 (61.5)	829 (62.6)	0.96 (0.57–1.59)	0.861
Living near the forest	29 (44.6)	599 (45.2)	0.98 (0.59–1.61)	0.921
Living in a house >30 m from the fields	23 (35.4)	477 (36.0)	0.97 (0.68–1.64)	0.916
Bathing in flooded fields	27 (41.5)	429 (32.4)	1.48 (0.89–2.46)	0.128
Fishing in flooded fields	43 (66.2)	797 (60.2)	1.29 (0.76–2.19)	0.339
Being age 20–29 y	12 (18.5)	266 (20.1)	7.31 (2.04–26.1)	0.002
Being age 30–39 y	8 (12.3)	226 (17.1)	5.73 (1.51–21.8)	0.010
Being age ≥40 y	42 (64.6)	346 (26.1)	19.7 (6.05–64.0)	<0.001
Being male	38 (58.5)	590 (44.6)	1.75 (1.06–2.90)	0.030

*SNV, Sin Nombre virus; Ig, immunoglobulin; OR, odds ratio; CI, confidence interval.

age and having killed rats in the field remained associated with hantavirus infection. In the model that did not consider sex and age, being a farmer (OR 2.63, 95% confidence interval [CI] 1.56%–4.41%) and having killed rats in the field (OR 2.30, 95% CI 1.38%–3.84%) were independently associated with hantavirus infection.

Hantavirus infection is endemic to the Maranhão western lowlands. It has characteristics of an occupational infection associated with agricultural work, but age mostly explained the effect of being a farmer (OR for being a farmer 1.29, 95% CI 0.74%–2.24%, adjusted for sex and age). Exposure to rodents appeared to occur both in fields and dwellings.

Although serologic evidence indicated that hantaviruses were circulating in the 6 municipalities studied, no cases of HCPS were reported in any of them. This finding suggests that either only mild or asymptomatic cases were occurring and that these were not recognized as HCPS or that cases of hantavirus were not being detected and reported. However, in a study in 2000 in Anajatuba in a cohort of 234 persons who did not initially have IgG against hantaviruses, 4 seroconversions to IgG against these viruses were detected; 2 of these infections were asymptomatic and 2 were self-limiting febrile illnesses that occurred after 24 months of follow-up (*9*). The present study reinforces the suspicion that mild or atypical cases are occurring in this region and may be the main reason classical hantavirus infection has not been identified.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Body Lice, *Yersinia pestis* Orientalis, and Black Death

To the Editor: Wild rodent fleas are the most common vectors of *Yersinia pestis*, the plague agent (*1*). The human body louse (*Pediculus humanus*) has been proposed as a probable additional vector during historical epidemics (*2*) because human cases of louse-borne plague have been suspected (*3*) and body louse–borne plague has been demonstrated experimentally with rabbits (*4*). Using rabbits, we tested the ability of the 3 *Y. pestis* main biovars to produce a successful rabbit-louse-rabbit-louse cycle of transmission (*4*).

Two New Zealand White (*Oryctolagus cuniculi*) rabbits were inoculated intravenously with phosphate-buffered saline alone (negative controls) or phosphate-buffered saline containing 10^9 CFU of *Y. pestis* biotype Nairobi-Rattus Antiqua, biotype 14–47 Medievalis, or biotype 6/69M Orientalis. PCR ensured detection of the virulence factor–encoding plasmids. The rabbits inoculated with biotypes Antiqua, Medievalis, or Orientalis had septicemia of $\approx 2 \times 10^3$ CFU/mL of blood 14 hours postinoculation and died at 20–22 hours, 18–20 hours, or 16–18 hours postinoculation, respectively. In contrast, the negative control rabbits remained healthy for 3 weeks. Five minutes postinoculation, 150 uninfected lice fed for 1 hour on rabbits and took an equivalent blood meal as measured by weight, regardless of the rabbit used. *Y. pestis* was isolated from all 120 randomly tested lice and their feces. Five days postinfection, the death rate of Orientalis-fed lice (95.3%) was significantly higher than that of the control (4%), Antiqua-fed, (78.6%), and Medievalis-fed (74%) ($p<0.0001$) lice. One third of Orientalis-infected lice remained alive 3 days after the contaminating blood meal.

Lice fed on septicemic rabbits further fed on 2 uninfected rabbits for 1 hour daily for up to 6 days. The rabbits bitten by Orientalis-infected lice had 2.7×10^2 CFU/mL of blood 4 days postinfection and died 1 day later. In contrast, the rabbits bitten by Antiqua-infected or Medievalis-infected lice looked healthy and lacked septicemia 3 weeks after challenge. New groups of 150 uninfected lice fed for 1 hour daily on Orientalis-infected rabbits started to die earlier than did lice fed on Antiqua, Medievalis (1 vs. 2–3 days after blood meal), and uninfected rabbits. Furthermore, 21 days after their first blood meal, lice fed on Orientalis-infected rabbits had a significantly higher death rate (90%) than did control (3%) ($p<0.0001$), Antiqua-infected (16%), and Medievalis-infected (10%) lice; the latter values were significantly higher than that of the negative controls ($p = 0.046$). *Y. pestis* could be cultured only from lice and their feces if the lice were fed on rabbits previously bitten by Orientalis-infected lice (online Appendix Figure, www.cdc.gov/EID/content/16/5/892-appF.htm).

Our observation that body lice effectively transmitted *Y. pestis* through a complete cycle of transmission confirms previous experimental (*4*) and field observations of experimental transmission that used body lice collected from plague patients from the same family in the absence of any other ectoparasite (*3*). Transmission of Orientalis but not Antiqua or Medievalis organisms did not result merely from experimental bias because negative controls remained negative, data were duplicated, rabbits exhibited equivalent bacteremia, and lice took equivalent blood meals regardless of biotype.

Our observations shed new light on the Black Death, a medieval epidemic of plague (*5*). Historical records indicate that persons with the Black Death had bubonic plague, indicating an ectoparasite-borne transmission (*1*). *Pulex irritans* fleas were documented in a medieval setting in Viking Greenland (*6*). However, their poor competence (*7*) and the Black Death that swept Russia and Scandinavia are not fully compatible with flea-borne transmission alone. Ten infected lice are sufficient for plague transmission (*4*), and our observation that one third of infected lice remained alive 3 days after infection indicates that an index plague patient carrying as few as 30 body lice could be a source for plague up to 3 days after dying. This figure was highly plausible during the Black Death because body lice currently infest almost 85% of homeless persons, with a mean of 57 lice per person (*8*). Although the role of fleas as vectors of *Y. pestis* from rodents to humans is undisputed, this tabulation sustains the potential role of body lice as an additional vector of plague from human to human during the Black Death (*2*).

Paleomicrobiology suggested that most historical cases in Europe resulted from Orientalis (*5*). This observation challenged the scenario that Antiqua, Medievalis, and Orientalis were responsible for ancient, medieval, and modern plague pandemics, respectively (*9*). The latter scenario had been hypothesized after the biotypes were observed to have a geographic repartition matching that of the hypothetical sources of the 3 historical pandemics (*10*) and was further propagated as dogma without further confirmation.

Our data support an alternative scenario of the historical plague epidemics transmitted by body lice, with Orientalis being the only such louse-borne transmissible biotype. This point justifies studies during ongoing epidemics in cold countries, keeping in mind the need to understand and control re-emerging plague in modern populations exposed to body lice.

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Salmonella Senftenberg Infections and Fennel Seed Tea, Serbia

To the Editor: The first documented outbreak of salmonellosis linked to consumption of plant products in the Autonomous Province of Vojvodina, Serbia, occurred from March 2007 through September 2008. Fourteen cases of *Salmonella enterica* serotype Senftenberg infection were reported.

The yearly incidence of salmonellosis in Vojvodina during 2003–2007 ranged from 25/100,000 inhabitants to 70/100,000 inhabitants; 34 outbreaks were reported in 2007, caused predominately by *S. enterica* serotype Enteritidis (*1*). Most outbreaks were associated with consumption of food of animal origin (*1*,*2*). *Salmonella* spp. were isolated from seeds in 2004, when *S. enterica* serotype Mbandaka and *S. enterica* serotype Virchow were isolated from sesame seeds (*3*).

Before 2007, *S.* Senftenberg had rarely been identified in Vojvodina. During 2003, 3 cases were reported. In 2004–2005, no *S.* Senftenberg cases were reported. In 2006, 8 cases of *S.* Senftenberg infection were reported among infants <12 months of age. An outbreak investigation did not reveal the source of infection. Common to all of those infected was their age and their consumption of infant formula. Nonetheless, laboratory analysis of samples of the various formulas did not show any pathogens. Two additional cases occurred in 2007 among patients who were <12 months of age. These cases confirmed suspicion that the infections had a source other than formula. Further investigation led to the consideration of tea consumption as a possible factor.

In April 2008, a total of 3 infants <12 months of age with salmonellosis came to the attention of investigators. *S.* Enteritidis was first identified in the samples of their feces. One month later, feces samples from the 3 infants were tested again, and *S.* Senftenberg was isolated from all 3 specimens.

After these findings, the Institute of Public Health of Vojvodina conducted an outbreak investigation in collaboration with institutes of public health at the district level. A case was defined as the presence of a laboratory-confirmed *S.* Senftenberg infection during 2007–2008. All case-patients (or their parents) were interviewed by using a standard questionnaire for salmonellosis, which was expanded to include questions regarding tea consumption.

A standardized method of enterobacterial repetitive intragenic consensus (ERIC)–PCR, based on the method of Versalovic et al. (*4*), with ERIC-PCR with ERIC2 primer (5′AAGTAA GTGACTCGGGTGAGCG-3′), was applied. DNA was isolated by using the InvitrogenPure Link Genomic DNA purification kit (Invitrogen, Carlsbad, CA, USA). Gene sequences were amplified in a Perkin/Elmer thermal cycler (model 9600) (PerkinElmer, Waltham, MA, USA). A DNA ladder was constructed by using Gene Ruler 100-bp DNA Ladder Plus (Fermentas, Glen Burnie, MD, USA).

Exploratory interviews with parents showed that all 3 infected infants had consumed commercially manufactured baby tea during the previous

month (after diagnosis of *S.* Enteritidis infection was made). Before feeding it to the infants, the parents had not heated the tea until it boiled, but rather had poured boiled water over the tea. After obtaining that information, we tested 33 samples of the incriminated brand of tea from public grocery stores and supermarkets; 13 samples were positive for *S.* Senftenberg. The organism's genetic profile was identical or similar from both tea and human samples (Figure).

Baby tea, widely distributed throughout Serbia, contains aniseed and caraway and fennel seeds. Sanitation inspectors collected samples from tea manufacturers. In the fennel seed sample, *S.* Senftenberg was identified. According to the tea manufacturer, fennel was purchased from another company, which collected seeds from individual producers. Fennel seed was cultivated in a household garden by an unregistered producer; neither the grower nor fennel stocks could be found. Two cases of *S.* Senftenberg from 2007 were retrospectively linked to infant tea, as were all other cases reported in 2008.

Demographic characteristics and clinical status of the case-patients were analyzed. Of 14 cases of *S.* Senftenberg infection, 10 were in infants <12 months of age (average 5.1 months). Half had diarrhea and the same proportion had fever >38.5˚C. Ten patients were female and 4 were male. All 4 adults had mild infection, except 1 adult who had concomitant *Clostridium difficile* infection. Three infants and an adult with concomitant infection were hospitalized.

Most infections were reported in May 2008, including the 3 cases in infants who were recovering from *S.* Enteritidis infection. After September 2008, no new cases of *S.* Senftenberg were reported until July 2009, when 1 case was identified in a 24-year-old man.

The heat resistance of *S.* Senftenberg is well known and is much higher than that for most other *Salmonella* serotypes (*5*). A number of recent outbreaks of *S.* Senftenberg infection resulted from consumption of fresh products. Thus, products that will be used in a fresh state should undergo more rigorous testing for pathogens, or better methods of infection control must be used.

The European Food Safety Authority has noted that all botanicals or botanical preparations could become hazardous as a result of flaws in the production process; therefore, manufacturers should follow the Hazard Analysis and Critical Control Point systematic approach (*6*). This system must be applied with the necessary flexibility and adapted to each botanical preparation on a case-by-case basis.

In 1999, the US Food and Drug Administration recommended that seeds be disinfected by washing with calcium hypochlorite solution before they sprout. However, this treatment destroys only pathogenic microorganisms on the seed surface (*7*,*8*). Thus, new methods, such as high hydrostatic pressure or use of bacteriophages as biocontrol agents should be adopted. High-pressure processing does not change the taste of food or cause any physical damage (*7*). With further refinement of phage delivery mechanisms, *Salmonella* phages could be effective in eliminating or reducing *Salmonella* contamination of vegetables (*9*).

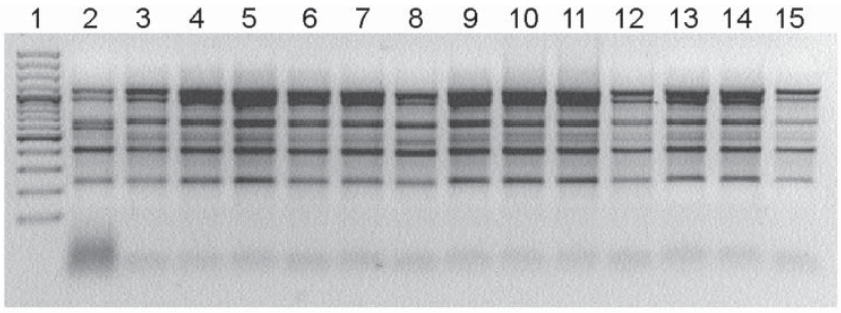

Figure. Enterobacterial repetitive intragenic consensus (ERIC)–PCR ERIC2 primers. Lane 1, molecular mass ladder; lanes 2–7, nonoutbreak isolates; lanes 8–9, isolates from baby tea; lane 10, isolate from fennel; lanes 11–15, isolates from salmonellosis patients. ERIC PCR with ERIC2 primer (5′-AAGTAAGTGACTCGGGTGAGCG-3′) was used. DNA was isolated by using the InvitrogenPure Link Genomic DNA purification kit (Invitrogen, Carlsbad, CA, USA). Gene sequences were amplified in a Perkin/Elmer thermal cycler (model 9600; PerkinElmer, Waltham, MA, USA). A DNA ladder was created by using Gene Ruler 100-bp DNA Ladder Plus (Fermentas, Glen Burnie, MD, USA).

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Cryptosporidiosis Associated with Wildlife Center, Scotland

To the Editor: Handwashing is the single most important prevention step in reducing transmission of gastrointestinal zoonoses (*1*). Nevertheless, Health Protection Scotland receives reports of 500 to 700 laboratory-confirmed cases of cryptosporidiosis each year. Cryptosporidiosis symptoms include profuse, watery diarrhea, often accompanied by bloating, abdominal pain, and nausea. On April 15, 2005, National Health Service Tayside District's public health department called a meeting of the incident control team after a single index case of cryptosporidiosis in Scotland. One reported case rarely results in such measures; however, initial investigations determined that this case-patient may have acquired infection by contact with scouring (diarrhea) lambs at a wildlife center, during the Easter break (March 27–April 10, 2005). Subsequent public health actions included active surveillance of recent *Cryptosporidium* spp. laboratory reports, active case finding, the microbiologic analysis of feces/rectal swabs from lambs and bedding samples, and an assessment of the wildlife center's private water supply. Control measures included the removal of lambs from the center, disinfection of the premises with hypochlorite, and stopping direct contact between animals and visitors.

In total, 128 microbiologically confirmed cases were reported to the incident control team. An additional 252 clinical cases were reported among wildlife center visitors for whom no stool sample was taken. The illnesses of these persons had a similar implied incubation period (typically 6–7 days) and their age profiles were the same as patients with laboratory-confirmed cases. Of 128 patients with confirmed cases, 117 visited the wildlife center, and infections of the remainder were attributed to secondary spread. Most case-patients were Tayside residents and were generally resident in towns and villages near the wildlife center. Of the 128 human isolates, 103 were identified as *Cryptosporidium parvum*. Oocysts from the environmental samples (lamb pen drain and central drain debris) were also identified as *C. parvum*. Isolates could not be obtained from lambs because the lambs had died and were subsequently incinerated by the wildlife center. Although assessment of the private water supply revealed unacceptable levels of coliforms, oocysts were not detected.

Daily gate receipts for the wildlife center were obtained. Using these as a denominator for confirmed cases, we calculated the daily attack rate. The attack rate peaked at 8.1% on April 8, 2005. The relative risk for visiting the wildlife center over the defined period was estimated to be ≈13.3 for confirmed *Cryptosporidium* infection. In view of the strength and clarity of the association between visiting the wildlife center (petting lambs in particular) and being a case-patient (Figure), no formal analytical epidemiologic investigation was conducted.

These results suggest that the outbreak was caused by direct contact with scouring lambs, a recognized risk factor for cryptosporidiosis, coupled with inadequate handwashing facilities (*2*,*3*). Anecdotal reports indicate that children were encouraged to pick up lambs from the farm enclosure, despite visible signs of diarrhea on the animals. The lack of handwashing facilities in this wildlife park was surprising because the Scottish government had conducted an information campaign that Spring (March), encouraging primary prevention initiatives, specifically in petting farms and zoos, and recommending the provision and use of handwashing facilities (www.infoscotland.com/handsclean/CCC_FirstPage.jsp). Moreover, no hand-

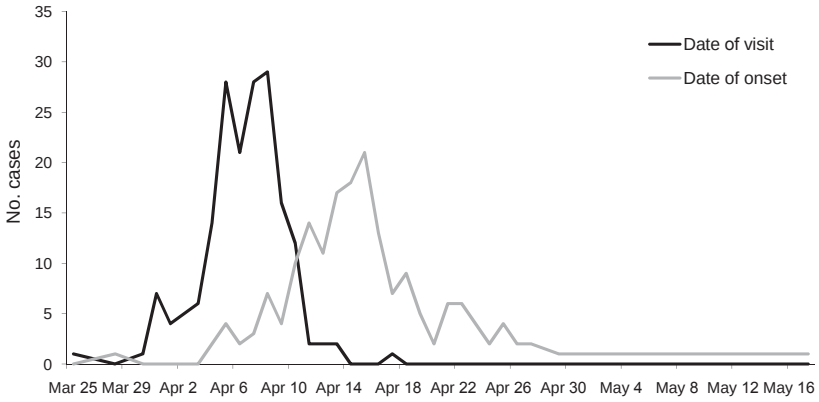

Figure. Date of onset of cryptosporidiosis cases reported to Health Protection Scotland and date of visit to wildlife center, 2005.

washing facilities were located near the lamb-petting area, and considerable effort was required to locate a handwashing basin in the wildlife center complex. Several alcohol hand sanitizers were located on site, but the microbicidal effects on *Cryptosporidium* spp. are insufficient to prevent infection, especially after direct contact with livestock (*4*,*5*).

After publication of the outbreak report, an assessment of hand-washing and hygiene facilities elsewhere in Scotland found them to be suboptimal and that stronger education, regulation, and other control measures were needed to protect the public. Recent *Escherichia coli* O157 outbreaks in England have accentuated the unresolved issues for UK petting farms concerning hand hygiene and zoonotic infections (*6*).

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Increase in Pneumococcus Macrolide Resistance, USA

To the Editor: Jenkins and Farrell reported an increase in the proportion of macrolide-resistant *Streptococcus pneumoniae* isolates in the United States (*1*). They mentioned increased use and inappropriate prescription of macrolides as potential explanations for the increase in macrolide resistance and expressed doubts, stating "which (if any) of these factors might explain the trends here are not clear." Although the spread of antimicrobial drug resistance is a complex issue with many contributing factors, we believe that the role of macrolide use should not be understated.

Several studies in Europe have provided evidence for a relationship between macrolide use and resistance. Macrolide exposure leads to emergence of macrolide resistance on the individual level, and countries in Europe with higher outpatient sales of macrolides have more macrolide-resistant pneumococci (*2*).

Outpatient antimicrobial drug use in the United States has decreased since 1995–1996, especially among children. However, use of azithromycin increased in children, and use of macrolides increased in older patients from 1995–1996 through 2005–2006 (*3*). In this context, it would be surprising that after this increase, pneumococci would show different characteristics in the United States than in Europe. A 2001 study showed that

increased macrolide use in the United States during 1995–1999 coincided with a doubling of the proportion of macrolide-resistant pneumococci (*4*), and further increases in macrolide use since 1999 (*3*) have contributed to the increase in macrolide-resistant pneumococci.

Decreased macrolide use has led to a decrease in macrolide-resistant pneumococci. A yearly seasonal reduction in antimicrobial drug prescribing in Israel was associated with a decrease in the proportion of antimicrobial drug–resistant pneumococci that caused acute otitis media (*5*). With the introduction of expanded-valent pneumococcal conjugate vaccines, there is promise that drug-resistant pneumococcal disease can be reduced. Nevertheless, judicious use of antimicrobial drugs and a decrease in unnecessary prescriptions, as promoted by the Get Smart: Know When Antibiotics Work (www.cdc.gov/getsmart) campaign, are essential to limiting selection and spread of antimicrobial drug resistance.

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Rapid Antigen Test for Pandemic (H1N1) 2009 Virus

To the Editor: Drexler et al. recently compared the sensitivity of the BinaxNOW Influenza A & B Rapid Test (BinaxNOW; Inverness Medical, Cologne, Germany) with that of a real-time reverse transcription–PCR (RT-PCR) assay specific for influenza A pandemic (H1N1) 2009 virus (*1*). Of 1,838 clinical specimens tested, 221 were confirmed as positive for pandemic (H1N1) 2009 by RT-PCR. When 144 of these 221 specimens were evaluated by using the BinaxNOW, results were positive for only 16 (11%).

At onset of the pandemic, we evaluated the first 135 nasopharyngeal aspirates submitted to the Regional Laboratory of Public Health Haarlem, the Netherlands. We compared the performance of the BinaxNOW for diagnosing influenza A (H1N1) virus by using molecular detection of influenza virus as the reference standard. Samples were analyzed with a general influenza A assay targeting the matrix gene (the RespiFinder assay) (PathoFinder B.V., Maastricht, the Netherlands [*2*]) and a pandemic (H1N1) 2009–specific RT-PCR assay targeting the neuraminidase gene (*3*). We tested 135 patient samples (76 from male patients); mean age of patients was 32 years (range 0–81 years). Samples from 38 (28%) patients had positive results in both RT-PCRs, and samples from 97 (72%) patients had negative results in the matrix gene RT-PCR and neuraminidase RT-PCR assays. Sensitivity and specificity were estimated to be 47% (18/38, 95% confidence interval [CI] 32%–62%) and 95% (92/97, 95% CI 88%–98%), respectively, for the BinaxNOW antigen test. Patients' ages did not significantly differ between rapid test–positive and –negative results.

Our results largely agree with those of Vasoo et al. (*4*) and the Centers for Disease Control and Prevention (*5*). Those studies determined that the sensitivity of the BinaxNOW compared with nucleic acid amplification tests is ≈40%. The lower sensitivity observed by Drexler et al. (*1*) might be because of differences in study type (retrospective evaluation compared with a prospective cohort in our study), sample size, technical factors (with regard to specimen collection, specimen transport, and specimen storage), differences in the test kit, and differences between individual patients (multiple categories of age and stages of illness, differences in virus shedding).

Many clinicians are not aware of the performance of specific test devices and rely on test results to make clinical decisions. Because negative results cannot rule out influenza, this test is of little use in a clinical setting without appreciation of the limitations of the test. However, because the BinaxNOW has reasonable specificity, it might prove useful in clinical or epidemiologic situations in which test sensitivity is not critical, e.g., in facility outbreaks in which multiple specimens are collected to rapidly identify the causative organism.

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In Response: We read with interest the report by Diederen et al. (*1*) showing a 47% sensitivity of the BinaxNOW (Inverness Medical, Cologne, Germany) antigen-based rapid influenza diagnostic test (RIDT) for the clinical detection of pandemic (H1N1) 2009 virus. We agree that RIDTs may be of little benefit in situations where a timely diagnosis by reverse transcription–PCR (RT-PCR) or optimized direct fluorescent antibody tests can be achieved.

Our recent study yielded even lower sensitivity for RIDT: 11.1% (*2*). RIDT sensitivity is greatly influenced by differences in the level of virus shedding between children and adults, making studies difficult to compare (*3*). In general, age profiles and virus concentrations should be provided and considered when comparing cohorts examined by any virus detection method. Moreover, quality and origin of specimens can influence the sensitivity of RT-PCR– and antigen-based tests. One important example is the use of flocked swabs for collecting respiratory samples. Under optimal conditions, for instance, a direct fluorescent antibody test was recently shown to yield high diagnostic sensitivity comparable with that of RT-PCR for pandemic (H1N1) 2009 virus (*4*). Another critical factor, especially for RIDT, may be the compatibility of test monoclonal antibodies with the novel virus. Lower sensitivities of such tests for pandemic (H1N1) 2009 virus in comparison with seasonal influenza viruses have been reported (*3*,*5*). Adaptation of RIDT antibody selection to pandemic (H1N1) 2009 virus may thus be necessary. Finally, we would like to emphasize the medical risks associated with use of RIDTs by untrained operators, e.g., lesions from inadequate sampling and false interpretation of test results. Such use may be specifically promoted by ready availability of such tests on the Internet or at pharmacies.

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Erratum—Vol. 16, No. 2

The link to the article Hendra Virus Outbreak with Novel Clinical Features, Australia (H. Field et al.) was published incorrectly in Vol. 16, No. 4. The correct link is www.cdc.gov/eid/content/16/2/338.htm.

The Evolution and Emergence of RNA Viruses

By Edward C. Holmes

Oxford University Press: New York, NY, USA, 2009
ISBN 978-0-19-921113-5 (Paperback)
Pages: 254; Price: US $53.83

This impressive monograph by Edward Holmes opens with a quotation from *La Peste,* by Albert Camus: "Everyone knows that pestilences have a way of recurring in the world; yet somehow we find it hard to believe in ones that crash down on our heads from a blue sky." This apt quotation might lead the reader to believe that the evolution and emergence of RNA viruses in causing new diseases would be discussed, but in fact the book, as its title suggests, concentrates on how RNA viruses evolve and emerge at the molecular level, not how they cause disease.

In addition to explaning what is currently known about the origins of RNA viruses, the book describes the mechanisms of RNA virus evolution, RNA virus quasispecies, and comparative genomics, as well as interesting new concepts, such as phylogeography. This term refers to the spatial movement of a phylogenetic species, which can be described in various ways (Holmes lists 5), two of which are the gravity model and the strong spatial subdivision model. In the former, patterns of transmission are driven by major population centers before moving out to smaller populations (influenza virus). In the spatial subdivision model, no clear evidence of migration among populations is presented (hepatitis C virus), and genomic diversity is partitioned into a series of clades (types and subtypes).

Holmes argues persuasively that research in this area is limited by the size and detail of genome databases, combined with relevant epidemiologic and clinical information, such as precise geographic location, exact date of sampling, and transmission dynamics of the disease. The book is fully referenced and has a useful index, and I recommend it to those who have knowledge of and interest in the molecular biology of RNA viruses.

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Sticky Decisions: Peanut Butter in a Time of *Salmonella*

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We present a consumer-focused perspective on creating communications regarding potentially contaminated foods. It is illustrated with decisions that might have faced US consumers during the 2009 recalls of peanut and pistachio products. The example shows how knowledge about test results and regulatory processes might be made more useful to consumers.

December 12, 2008

A prudent, informed consumer is about to open a jar of peanut butter. It is one of her favorite foods. Mostly, she loves the taste, but she also knows it as a cheap, healthy food—although she is a little fuzzy on those details (*1*–*3*). Like most Americans, she always has peanut butter at home. Unlike most, though, she always considers the risk for *Salmonella* infection, before opening a new jar, then decides whether to eat it, toss it, or wait a month to see if any problems turn up. She has done some research too. Here is her reasoning.

If she eats the peanut butter and gets salmonellosis, then she has to pay for treatment and lose work time. The US Department of Agriculture (USDA) estimated that an average case in 2007 cost $1,821 in lost wages and medical costs (*4*). She can imagine her case costing less (if her insurance covers the medical costs and she uses otherwise "wasted" sick-leave days) or more (if the opposite is true), but she decides to use $1,821 in her decision making.

If she throws out the peanut butter, she will lose its $3 cost. Getting a refund is such a hassle that it will still feel like losing $3.

If she waits a month, she will incur the opportunity cost of the money tied up in the peanut butter. She puts that at $0.02 (using 8% annual percentage rate).

Now, she just needs to know the probability of salmonellosis. If it is greater than 1/607, then she should toss the jar, comparing its cost ($3) with that of getting sick ($1,821). If it is greater than 1/91,050, then she should wait a month, comparing its cost ($0.02) with that for getting sick ($1,821), assuming that food inspectors find any problem by then, which makes the risk zero.

But money isn't everything.

If she throws out the jar, getting another will be a small hassle. She decides that the $3 covers that. She would feel bad about wasting the food but also feel good about her prudence. So, those psychological effects balance out. She vaguely worries that the same logic will lead her to throw away the next jar (and the next). That *would* make her feel bad.

If she waits a month, then the peanut butter might lose taste or nutritional value, or somehow "go bad." However, she can't find any good information about those possibilities and decides to ignore them. It is just a month.

Therefore, if she eats from the jar, the only important nonmonetary consequence is her getting salmonellosis. She knows that it usually involves an illness of 4–7 days, with diarrhea, fever, and abdominal cramps, and that most persons recover without treatment. However, the diarrhea sometimes calls for hospitalization, and the infection can spread to the blood. People can die, if not treated promptly with antimicrobial drugs (*5*). Even if USDA has not put a dollar value on suffering, she would pay a lot to avoid it. She would pay even more if she had, or was making the decision for someone with, a weak immune system.

If she tosses the jar, then she will face the risk of driving to the store to get it. She puts that at 1 chance in 100,000 of an accident, and 1 chance in 50 of that crash being fatal (*6*).

If she eats from the jar and it contains any *Salmonella* bacteria, she will probably consume some of the bacteria, given how peanut butter is made. She realizes that she can put an upper boundary on that risk: in 2007, a total of 1 in 6,702 Americans contracted foodborne salmonellosis, from all sources (*7*). Given that most Americans eat peanut butter, her chances must be smaller—unless there are problems.

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That probability is much less than the 1/607 threshold. Therefore, based on purely economic considerations, there is no point in tossing the jar, even if she considers the suffering that USDA ignored. That probability is higher than the 1/91,050 threshold for waiting. But 1/6,702 is such a conservative estimate and there are so many nonmonetary reasons not to wait—and the peanut butter looks so good.

She knows the US Food and Drug Administration (FDA) montiors food safety, and she checks its website for recall notices (*8*). No reported problems! So, she opens the jar and enjoys the great taste of peanut butter.

January 12, 2009

A month later, she buys a new jar. The next day, her morning web check finds that FDA, the Centers for Disease Control and Prevention, USDA, and others are investigating a multistate outbreak of *Salmonella enterica* serovar Typhimurium infection, and peanut butter is the likely source. FDA is inspecting an unnamed manufacturer and tracing its distribution channels.

Looking at her new jar, she realizes that only one thing has changed since her last decision: the probability of contamination. It must be larger, but by how much? The announcement says nothing about that probability and provides no advice. She wonders what that means. Is the outbreak under control? Are they waiting for authoritative information? Is it up to the firm to issue a recall?

Without a clear signal, she opens the jar. The peanut butter tastes as good as ever, but she does not enjoy it as much. In fact, she is so troubled about what she has just eaten that she expands her online search beyond her daily visit to the recall website. She realizes that she can't undo her exposure. However, perhaps she can get some reassurance—or faster medical attention, if need be.

The recall website mentions no product names. However, her Google search (on "peanut butter," "Salmonella," and "multistate outbreak") shows that, 2 days earlier, King Nut Company voluntarily recalled peanut butter manufactured by Peanut Corporation of America (PCA) and distributed under its King Nut and Parnell's Pride labels. King Nut says that these brands are only sold wholesale and that all its other products are safe. Although she eats another brand, she keeps worrying.

How confident can King Nut be about its other brands? Are none of the peanuts grown in the same fields, shipped in the same trucks, processed at the same facilities, or handled by the same employees? Are other companies doing their own inspections? Can FDA require tests and recalls? How good, and fast, are the tests? Without answers to these questions, what has been found does not tell her what might be found.

Over the next month, she has good news: she does not get sick. She also has bad news: seeing the outbreak reports explode on her daily website checks. Although she is fine physically, she feels like she has dodged a bullet. She cannot understand the 2-day lag between the announcements by the government and by King Nut, which she kicks herself for having missed. Had she gotten sick, would she have found the information that she needed to get timely treatment?

February 12, 2009

It's time to buy more peanut butter. However, a lot has happened during the last month. On January 13, PCA announced a voluntary recall of 21 lots of peanut butter and peanut paste produced in its Blakely, Georgia, USA, facility. By January 27, it had expanded the recall 3 more times. The next day, PCA recalled all dry- and oil-roasted peanuts and peanut products processed at Blakely since January 1, 2007. On January 30, FDA confirmed reports of a criminal investigation of PCA for continuing to ship products after receiving positive *Salmonella* test results. Hundreds of persons are sick; 9 die. The case-fatality rate is about that of previous outbreaks, which suggests that the strain is not unusual, although the scope is.

The recall website now provides consumer recommendations and a searchable database, for recalled products. Her favorite brand is not on the list, so she still believes that her risk is negligible. She makes her usual monthly purchase, then has a moment of truth when she gets home: she still does not know how anyone decides which products to test or what information to share. That 2-day lag still bothers her, as does wondering when the criminal investigation began. She is unhappy about her "no news is good news" inference, last month, and maybe this one, too.

She is devoted to FDA's websites: however, she also knows that there are limits to its resources and legal authority. She just doesn't know what they are. So, she makes some guesses.

Given the stream of new recalls, she concludes that FDA waits for strong positive evidence before saying anything. As a result, she can't tell whether her favorite brand has been cleared or just not yet tested. Her rule is still to toss a jar if the risk is over 1/607. The recall list currently has ≈50 peanut products. There would have to be over 30,350 (50 × 607) peanut products, for the rate to be under her threshold. She should toss the jar if there is a similar rate among products that have not been officially cleared.

But what does she know? Maybe she should be worried that other foods are processed, shipped, or shelved along with the peanut butter. She knows that life has risks and she is willing to take reasonable ones. But she hates not knowing what's going on.

In the following days, the number of salmonellosis cases increases, confirming her fears. Still, there is no recall for the major national brands, including her own. That's

good. She just doesn't know how good without understanding what gets tested and announced.

At least she can eat her second favorite nut product, pistachios.

March 12, 2009

Her month of watchful waiting has passed. Her morning website checks have found a continuing but slackening stream of recall notices and salmonellosis cases. FDA reports conducting more audits and inspections and collaborating with other authorities. It still has found no contamination in major national brands. Although that message has not changed, she assumes that the supporting evidence is now stronger. Still, she is unnerved enough not to open her jar or to buy other peanut products, just in case she has missed something. She'll stick with pistachios.

During the month, she had a disquieting experience: She returned a box of peanut butter granola bars. The merchant refunded her money, no questions asked. However, she found that the product was still on the shelves, while her favorite granola bar, with the tiny chocolate chips, was missing. She guesses that the store was humoring her, by giving a refund for a safe product, while peanut butter was a micro ingredient in the chocolate chip bar. The refund gets her wondering whether food manufacturers are more careful with products featuring peanut butter, compared to ones where it is a trace ingredient (or an "industrial chemical"). She hopes that qualified people worry about these things. She just wants useful information.

March 31, 2009

Her morning web check reveals the shocking news that the second-largest US pistachio processor (Setton) has voluntarily recalled certain lots of roasted nuts. She never worried about pistachios before. However, if they can be contaminated, then she faces the same decision as with the peanut butter. The cost is even the same. To control her passion for pistachios, she buys small ($3) packages.

She has a harder time figuring out the risks. The announcement advises that "Consumers should not eat pistachios or food products containing them until they can determine that the products do not contain pistachios recalled by Setton." How can she tell? Are all those products sold under the Setton name? What does it mean that untested pistachios are suspect, whereas only tested peanuts were? Are the risks that different? Perhaps pistachio and peanuts are processed differently. Perhaps Setton is less trustworthy than PCA (criminal investigation notwithstanding). Perhaps the authorities know more than they are allowed to reveal. Perhaps the reporting policy has been changed. If so, how? Are they being hypercautious? Can she then be totally confident about almonds, her third favorite nut? Or, are they being so cautious that everything will soon be suspect? If so, perhaps she should just eat those pistachios.

April 12, 2009

A month has passed without her favorite brand appearing on the recall list. She is about to open the jar, when her morning Internet check finds that a company named Westco/Westcott has "declined" FDA's request to recall its products with PCA peanuts and "to provide access to certain records about the distribution of these products." In response, FDA has asked US Marshals to execute an inspection warrant (*9*). She is shaken to learn that FDA cannot require food recalls. Realizing how little she knows about FDA's authority and resources, she decides to let her poor jar sit for another month.

What Does She Want?

One morning, she notices a "Contact Us" option at the recall website. She thinks, "What do I want? I know that they're working hard to protect me. But, somehow, I'm not quite getting the information that I need to protect myself. I like the notices' standard format. I know how to find things on it. I've learned to decode most of the jargon. I just don't know what it all means in terms of my risks. Maybe if I grade some of the postings, it will clarify my thinking":

January 12 (*10*). Grade: D. Although I learned that there was an outbreak, possibly related to peanut butter, I did not learn anything about what to do, even though the King Nut recall was already happening. Seeing how complex the peanut production system is, I feel like they must have suspected that they had not found all the problems. They could have said, "We recommend not eating peanut products until we do more testing. Don't throw them away, though; they may be fine." They could also have said that they cannot force recalls or do all the testing that they want, so that I would know what they are up against.

January 16 (*11*). Grade: B. By saying that they could not say which brands to avoid, they allowed me to make a better decision. Still, it was unnerving to see such a big change, over 4 days, without an explanation why.

January 17–19 (*12–14*). Grade: B+. They recommended the decision that I would have made on my own, had I known what they knew. Still, I was left wondering about the PCA controversy and what it meant about future surprises. It would have helped just to hear, "We cannot comment on the ongoing criminal investigation of PCA."

After thinking through these dates, she knows what she wants and thinks that it wouldn't be too hard to do:

- *Talk to consumers*. Find what decisions we face and what we worry about. I am cooking for one person and can afford to wait. Other people can't.

- *Tell us what you know—and don't know*. We would like you to be certain but don't want to learn too late about possible problems.
- *Tell us if your hands are tied*. If we expect you to do the impossible, then we'll get mad at you, not at those who keep you from protecting us.
- *Get the information that we need*. Do the tests that will tell us what not to eat. We don't care that much about "all clears."
- *Have some ordinary people read each message before you post it*. If they understand it, then most everyone will.

Some of what she wants is happening already. For example, in January 2010, FDA established a new website, FDA Basics, to inform consumers about how FDA works (*31*).

Coda

Our hypothetical consumer is unusually, but not implausibly, thoughtful about her food safety decisions (and her love of peanut butter), given the picture that emerges from the large research literature on the topic (*15*,*16*). Although consumers' safety behavior is often disappointing, some of those failures reflect their difficulty understanding what to do (*17*–*23*). Risk communication research often finds large gaps between what experts say and what consumers hear—and need to hear. Fortunately, research can close much of that gap, allowing public health officials to do all that is possible to help people to make wise choices in an uncertain world (*24*–*31*).

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Judith Leyster (1609–1660) ***Boy Playing the Flute*** **(1630–1635)** (detail) Oil on canvas (73 cm × 62 cm) The National Museum of Fine Arts, Stockholm, Sweden

The Whole Heaven a Musical Scale and a Number

Polyxeni Potter

A woman ahead of her times, Judith Leyster was born the last child, a prodigy, in the large brood of a cloth maker and brewer in Haarlem. While still in her teens, she earned favorable mention in Description and Praise of the City Haarlem in Poetry, a book by Dutch poet Samuel Ampzing. She received art training, probably from Frans de Grebber and may have worked in the studio of well-known portrait painter Frans Hals. In her early 20s, she was admitted into the Haarlem painters' Guild of St. Luke, soon had her own studio and apprentices, and earned a living as master painter. Her work—mostly portraits and genre scenes filled with merriment—was influenced by the Utrecht school, the followers of Caravaggio, who adopted the Italian master's use of chiaroscuro. Despite her accomplishments and a measure of fame during her lifetime, Leyster was forgotten after her death at age 50, many of her works mistakenly attributed to other artists. She was rediscovered toward the end of the 19th century. Few of her paintings have survived.

Leyster was innovative, not only in her range of subjects, which was broader than that of her contemporaries, but also in her interpretation of genre. Beyond the usual tavern scenes popular with the buying public, she favored women at home, not a usual theme in Holland until the 1650s. Her view of Dutch domesticity was informal and engaging, subtle and intimate; her use of light and shadow dramatic. Her subjects displayed emotion, liveliness, and a confidence and flair that extended to the distinctive monogram of her signature, the letter "J" capped with a star (lode star or lead star)—a playful reference to her last name.

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Genre scenes provide a thorough look into all aspects of the 17th century; music for one, which was ubiquitous in both life and art. Since no recordings were available, music was often spontaneous and always live in homes, taverns, or the outdoors. Scenes containing dance or other amusements always featured musicians and their instruments. Music was woven into the fabric of society and lent itself comfortably to teaching moral ideals so vital to the prevailing Calvinism: "As the old have sung so pipe the young." Musical instruments were common too in artists' studios. Painters struggling to raise the social rank of their profession above craft used them to show their understanding of music and solidify their connection with the liberal arts.

Music's power to inspire and animate originated in antiquity with the writings of Pythagoras, who discovered the physical relationship, expressible in ratios, between mass and sound. "[The Pythagoreans] saw that the … ratios of musical scales were expressible in numbers [and that] … all things seemed to be modeled on numbers, and numbers seemed to be the first things in the whole of nature, [they] supposed the elements of number to be the elements of all things, and the whole heaven to be a musical scale and a number." Accordingly, the distances between planets would have the same ratios as produced harmonious sounds in a plucked string. The stars and planets, rotating en masse, would produce cosmic harmonies, termed music of the spheres, not accessible to the human ear.

Artists, perhaps intrigued by this celestial connection, have often painted musicians with their eyes lifted upward as a sign of spirituality. Instruments were often used as symbols: strings associated with Pythagorean harmony, wind instruments the province of shepherds and peasants. One of the most musical of her artistic contemporaries, Leyster

showed her love of instruments and their sounds in many of her works. She painted them with virtuosity within the recurring theme of music, though she took liberties with the usual classification, marrying such antipodes as the violin and recorder or linking inspiration with the flute.

In *Boy Playing the Flute*, on this months' cover, she expertly painted several instruments, along with the performance of a young musician. Balance and harmony in the scene are achieved not by the single figure alone playing the transverse flute but by an ensemble, two of the instruments hanging prominently on the wall beside him, a violin and a recorder. The violin, with its elongated sound holes and exaggerated points, bow threaded behind the strings and no chin rest, seems a fine period specimen, as does the recorder, with its strikingly long windway and smoothly convex foot. The recorder was signed, according to convention, near the mouthpiece by its maker, Leyster. The instrument held reverently by the boy is a Renaissance flute cross-blown and stretched diagonally across the painting. Hues of carob and honey in the clothing and instruments radiate warmth on to the pock-marked wall.

The boy sits awkwardly gazing toward the light. Like all children of this period, he is dressed with the sobriety and modesty of a small adult in velvet coat, linen collar, and a deep red hat, the only relief in the drab attire. His sleeve has been discreetly darned at the elbow, and the chair has seen better days, its painted post discolored and back slat broken, causing him to slouch. Declining affluence aside, this boy is in comfortable enough circumstances. He seems focused on the task, rapt even, as he pushes air through the mouth and manipulates it with slender sensitive fingers to make music. His face, framed between the red hat and white ruff collar is a beacon. His earnest expression belies the wind instrument's lowly associations.

During this its golden age, Holland's children were not much better off than most of their other European counterparts, even though progress was made in some areas that affected their survival. The age saw a rebirth of medicine, and for the first time since Hippocrates, clinical observations became important. Influenza, chorea, scarlet fever, scrofula, and pertussis were recognized diagnoses, even though we now know they are not all specific diseases. Franciscus Sylvius, Dutch physician and scientist, identified characteristic changes in the lungs caused by consumption, rampant at the time.

Yet childhood was hardly celebrated as a stage of life, marred as it often was by poverty, social inequity, and high death rates, especially from infectious diseases. Children were viewed as unfinished creatures to be raised into adults. Parenthood was a public virtue, and a basic education, including private music-making for devotional or recreational purposes, was thought good for moral development. Nonetheless, by age 10, most boys were transferred to a master to learn a trade, and poor or orphaned children had already entered the labor force.

Leyster and some of her contemporaries portrayed children sympathetically, allowing them expressions and emotions, music and art, at a time when many of them did not survive their early years. The child in *Boy Playing the Flute* has fallen on hard times. He is teetering between poverty and comfortable domesticity. Though he still has a home with a violin hanging on the wall, there is no music master in the room. He is going it alone. But "You sing what you hear," and he hears the harmony above.

While this young musician navigates changing fortunes, he still has an advantage over many of his contemporaries. He is healthy, having so far escaped the scourges of his times, though impending poverty undoubtedly puts him at risk. Pneumonia, diphtheria, diarrheal diseases, tuberculosis, and streptococcal infections were major causes of childhood illness and death, as they still are in much of the world. Some, among them respiratory infections that might have cut the youth's flute career short, are now under control because of expansive vaccination programs, including in today's Netherlands.

But while a young boy might tab into the music of the spheres for inspiration and survival, those who try to break the axiomatic connection between wealth, poverty, health, and illness can only rely on programs aimed at the great killers of children. Persistent and unrelenting disharmony, caused by diseases upon diseases emerging and reemerging amidst endless complicating factors, interferes in public health with their orderly conquest, one by one, in any kind of anticipated pattern or ratio on a cosmic scale.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Increased Prevalence of *Trichinella* spp., Northeastern Germany

Increase of PI-2 Pili in *Streptococcus pneumoniae*

Rift Valley Fever, Madagascar, 2008–2009

Evolutionary Histories of *Borrelia burgdorferi*, Northeastern and Midwestern United States

Oseltamivir-Resistant Influenza Viruses during 2007–2009 Influenza Seasons, Japan

Novel Norovirus Associated with Diarrhea in Dogs

Causes of Infection after Earthquake, China, 2008

Dengue Virus 3 Genotype I in *Aedes aegypti* Mosquitoes and Eggs, Brazil, 2005–2006

Human Respiratory Enterovirus Genotype EV-104, Italy

Pandemic (H1N1) 2009, Shanghai, China, May–July 2009

Transfer of Plasmid Encoding *Klebsiella pneumoniae* Carbapenemase 3 to *Escherichia coli*

Pneumovirus Isolated from Dogs with Acute Respiratory Disease

Genetic Evidence of Tacaribe Serocomplex Virus, Mexico

Pulsed-Field Gel Electrophoresis in Surveillance of *Salmonella* Infection, Texas

Identification of Betaherpesvirus in Bats

Vaccinia Virus Infection in Monkeys, Brazilian Amazon

Whence Feral Vaccinia?

Atypical Chikungunya Virus Infection in Immunocompromised Patients

Complete list of articles in the May issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

May 20–22, 2010
VIII International Conference on HFRS, HPS and Hantaviruses
Athens, Greece
http://www.hantavirusconference2010.gr

June 7–8, 2010
Research Advances in Malaria: Biology of Mosquito Vectors
Johns Hopkins Bloomberg School of Public Health
Baltimore, MD, USA
http://malaria.jhsph.edu/events/2010/vector_biology/index.html

June 8–11, 2010
Asia Infectious Diseases Forum 2010 (Part of World Vaccine Congress Asia 2010)
Grand Hyatt, Singapore
http://www.terrapinn.com/2010/infectious

July 11–14, 2010
International Conference on Emerging Infectious Diseases 2010
Hyatt Regency Atlanta
Atlanta, GA, USA
http://www.iceid.org

August 28–September 1, 2010
Infectious Disease 2010 Board Review Course – 15th Annual Comprehensive Review for Board Preparation
Hyatt Regency Crystal City
Arlington, VA, USA
http://www.IDBoardReview.com

November 11–13, 2010
European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)
Lisbon, Portugal
http://www.escaide.eu

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **www.medscapecme.com/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.com. If you are not registered on Medscape.com, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Tropheryma whipplei in Children with Gastroenteritis

CME Questions

1. Which of the following statements about the epidemiology of *Tropheryma whipplei* gastroenteritis in the study cohort is most accurate?

A. 15% of children with gastroenteritis have positive testing for *T. whipplei*
B. The infection rate with *T. whipplei* increased gradually from 2006 to 2008
C. Infection with *T. whipplei* was most common during the winter months
D. There were significant rates of positive *T. whipplei* testing in children without diarrhea

2. Which of the following statements about the bacterial loads of children with positive testing for *T. whipplei* is most accurate?

A. Bacterial loads were undetectable or low in the majority of children
B. Bacterial loads were lower than that of chronic carriers
C. Bacterial loads were comparable to those of individuals with Whipple's disease
D. Bacterial loads generally remained elevated after the resolution of diarrhea

3. Which of the following statements about laboratory test results in children with *T. whipplei* gastroenteritis is most accurate?

A. One genotype of *T. whipplei* was associated with all cases
B. Co-infection with other pathogens was more common in patients with *T. whipplei* gastroenteritis compared with other children with diarrhea
C. There was no difference in the rate of seropositivity for *T. whipplei* in comparing cases and controls
D. Co-infection with other pathogens was limited to children with higher bacterial loads of *T. whipplei*

4. The following are clinical features of *T. whipplei* gastroenteritis compared with other infectious diarrhea, except:

A. Shorter duration of hospitalization
B. Shorter duration of fever
C. Less anorexia
D. Smaller degrees of weight loss

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

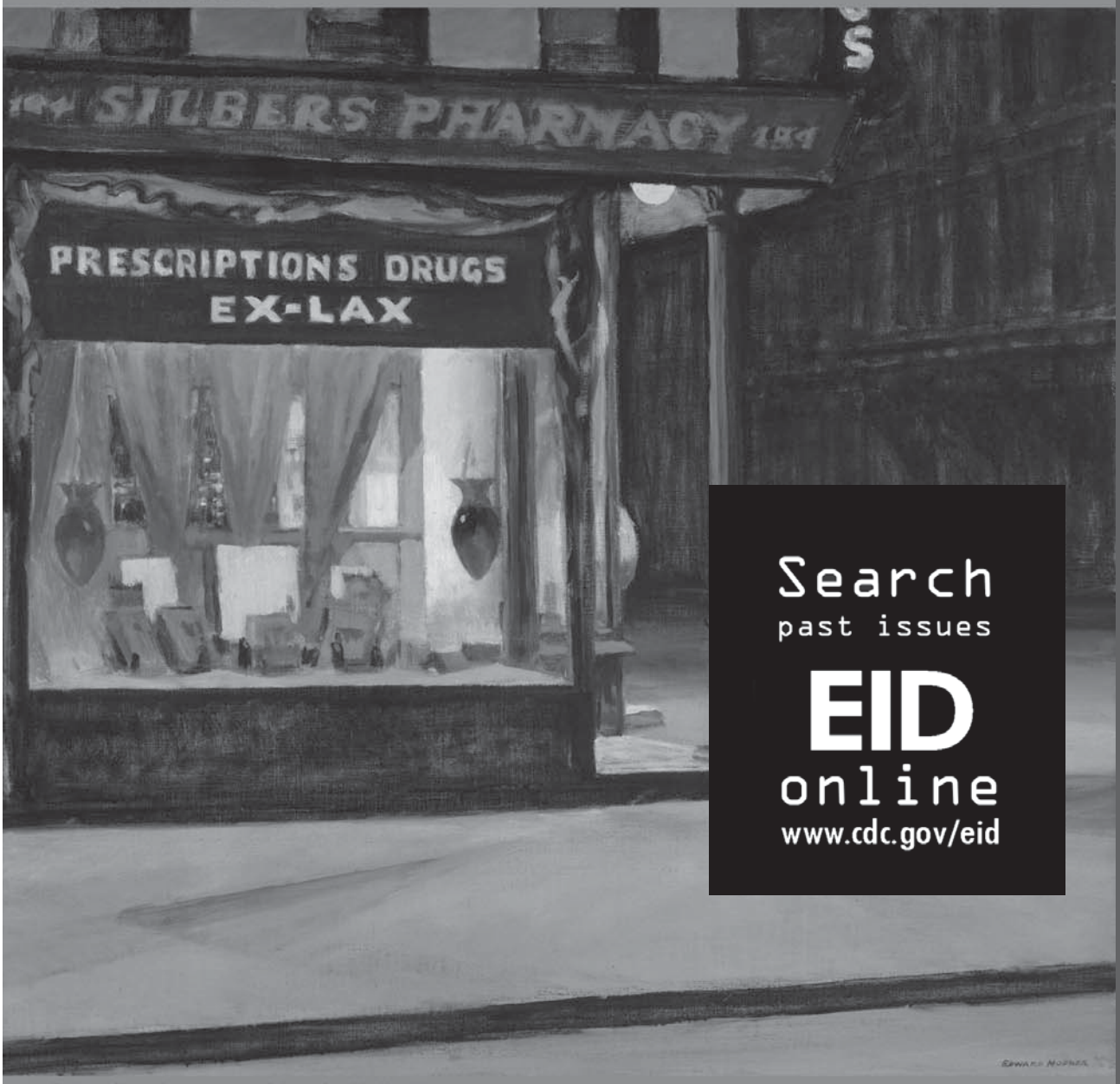
2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

EMERGING INFECTIOUS DISEASES®

April 2010

Enteric Infections



The Museum of Fine Arts, Boston, Massachusetts, USA, Bequest of John T. Spaulding, 48.564

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles see below and visit www.cdc.gov/eid/ncidod/ EID/instruct.htm.

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Instructions to Authors

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, figure legends, appendixes, and figures. Each figure should be in a separate file.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

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Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

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